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AUTHOR – AUTEUR

Full Name of Author – Nom complet de l'auteur

Glen Douglas Wheeler

Date of Birth – Date de naissance	Canadian Citizen – Citoyen canadien
March 8, 1958	☑ Yes / Oui ☐ No / Non
Country of Birth – Lieu de naissance	Permanent Address – Résidence fixe
Canada	10552 – 66 Ave Edmonton, Alberta T6H 1X4

THESIS – THÈSE

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Univ. of Alberta	Dr. M. Schachter

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IMMUNOLOCALIZATION OF KALLIKREIN IN VARIOUS TISSUES

by

GLEN D. WHEELER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled IMMUNOLOCALIZATION OF KALLIKREIN IN VARIOUS TISSUES submitted by GLEN D. WHEELER in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

M Schachter
.....
Supervisor

R Kinsman
.....
Susanne Barton
.....
E J Sanders
.....
Ronald Worthington
.....
D Murray Poole
.....
External Examiner

Date Sept. 27, 1985

DEDICATION

To My Parents

ABSTRACT

Many techniques were employed for the immunocytochemical localization of tissue kallikreins (EC 3.4.21.35) and nerve growth factor (NGF). Freeze-drying and chemical fixatives were successful but to varying degrees. Resin-embedded tissues were better preserved and yielded better results than paraffin embedding. Two methods of immunostain were most successful, namely peroxidase-antiperoxidase (PAP) and avidin-biotin complex (ABC).

Tissue kallikrein was immunocytochemically localized in various mammalian tissues, including the submandibular gland of cat and guinea-pig where it was localized in apical regions of striated duct cells. It was also localized in the goblet or mucous cells of the colon and in the mucous cells of the stomach of the cat and rat. At the ultrastructural level, it was localized in the small apical secretory granules of striated duct cells of the submandibular gland in cat and guinea-pig. Kallikrein was also immunolocalized in surface epithelial secretory cells of the guinea-pig coagulating and prostate glands; also in secretory cells of the submucosa of cat trachea. Nerve growth factor (NGF) was immunolocalized in the surface epithelial cells in the guinea-pig prostate gland.

The biological and evolutional significance of the broad group of serine proteases is discussed. It is suggested that these enzymes play multiple and diverse roles in bioregulatory processes, and these various possibilities are considered and discussed.

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Table of Contents

Chapter — Page

I. INTRODUCTION 1

A. Kallikrein-Kinin System 1

1. Salivary gland 2

2. Pancreas 6

3. Colon 7

4. Intestine 8

3. Stomach 9

6. Kidney (Urinary) 9

7. Respiratory Tract 12

8. Male Sex Glands 12

9. Brain 13

10. Other Tissues 13

B. Nerve Growth Factor 14

II. MATERIALS AND METHODS 17

A. Animals, Organ Dissection, Tissue Preparation and Extracts 17

B. Fixation of Tissues 18

1. Chemical Fixation 18

2. Freeze-Drying 22

C. Embedding 23

D. Immunocytochemistry 26

1. Antisera 26

2. Immunostaining systems 29

a. Peroxidase-antiperoxidase 29

i. Paraffin sections 29

ii. Semi-thin resin sections 32

iii. Ultrathin resin sections 33

b. Avidin-biotin complex 35
i. Semi-thin resin sections 35
ii. Ultrathin resin sections 38
iii. Pre-embedding 39
c. Protein A-colloidal gold 40
i. Ultrathin resin sections 40
3. Specificity and controls 40
E. Microscopy and Photography 41
III. RESULTS 42
A. Summarized Results of Immunostaining 42
B. Localization of Kallikrein 42
1. Submandibular Gland - Light and Electron Microscopy 42
a. Cat 42
b. Guinea-pig 57
2. Colon 57
a. Cat 57
b. Rat 67
3. Stomach 72
a. Cat 72
b. Rat 81
4. Respiratory Tract 84
a. Cat 84
C. Localization of Kallikrein and Nerve Growth Factor 91
1. Male Sex Glands - Light Microscopy 91
a. Guinea-pig 91
IV. DISCUSSION 103
A. Kallikrein and Related Proteases in Salivary Glands 103

B. Kallikreins, Nerve Growth Factor and Related Proteases in Male Sex Glands and Their Possible Roles 106

C. Gastrointestinal Kallikreins and Their Possible Roles 107

D. Interpretation of Immunocytochemical Findings 108

E. Possible Sources of Circulating Tissue Kallikrein 111

V. CONCLUSION 112

VI. REFERENCES 113

LIST OF TABLES

Table	Description	Page
1	Summarized results of light microscopic immunostained tissues fixed in various ways	43

LIST OF FIGURES

Figure	Description	Page
1	Low temperature freeze drier and copper block freezing device	24-25
2	Double diffusion analysis of αCSK with cat colon and cat submandibular gland	27-28
3	Summarized PAP method	30-31
4	Summarized ABC method	36-37
5	Kallikrein localization in striated duct cells of the cat submandibular gland (paraffin sections)	44-45
6	Control section for Figure 5	44-45
7	Kallikrein localization in apical regions of striated duct cells of the cat submandibular gland (semi-thin sections)	46-47
8	Control section for Figure 7	46-47
9	Electron microscopic localization of kallikrein in apical secretory granules of the cat submandibular gland, striated duct cells	49-50
10	Control section for Figure 9	49-50

11	Ultrastructure of apical region of striated duct cells fixed by strong aldehyde fixative	51-52
12	Ultrastructure of apical region of striated duct cells fixed by weak immunocytochemical fixative	51-52
13	Localization of kallikrein in apical granules, stained as in Figure 9, fixed as in Figure 12	51-52
14	Kallikrein localization in apical regions of striated duct cells of the cat submandibular gland, post-fixation with osmium (semi-thin sections)	53-54
15	Control section for Figure 14	53-54
16	Electron microscopic localization of kallikrein in apical secretory granules of the cat submandibular gland, striated duct cells, post-fixed with osmium	55-56
17	Control section for Figure 16	55-56
18	Electron microscopic localization of kallikrein in apical secretory granules of the guinea-pig submandibular gland, striated duct cells	58-59
19	Control section for Figure 18	58-59
20	Kallikrein localization in mucous cells and secretions of the cat colon (paraffin sections)	61-62
21	Control section for Figure 20	61-62

22	Kallikrein localization in mucous cells and secretions of the cat colon (semi-thin sections)	63-64
23	Control section for Figure 22	63-64
24	Kallikrein localization in mucous cells and secretions of the cat colon (freeze-dried tissue, semi-thin sections)	65-66
25	Control section for Figure 24	65-66
26	Electron microscopic localization of kallikrein in the mucous granule region of cat colon mucous cells	68-69
27	Control section for Figure 26	68-69
28	Kallikrein localization in mucous cells of the rat colon	70-71
29	Control section for Figure 28	70-71
30	Kallikrein localization in apical regions of mucous cells of the cat stomach antrum (osmium post-stain)	73-74
31	Control section for Figure 30	73-74
32	Kallikrein localization in apical regions of mucous cells of the cat stomach antrum (without osmium post-stain)	75-76
33	Control section for Figure 32	75-76

34	PAS stain with immunostain for kallikrein in cat stomach antrum (high magnification)	77-78
35	Kallikrein localization in periphery of mucous cells of cat stomach antrum	77-78
36	Control section for Figures 34, 35 and 37	77-78
37	PAS stain with immunostain for kallikrein in cat stomach antrum (low magnification)	77-78
38	Kallikrein localization in mucous cells of cat stomach antrum (PAP method)	79-80
39	Control section for Figure 38	79-80
40	Kallikrein localization in mucous cells and secretions of cat stomach antrum (ABC method)	79-80
41	Control section for Figure 40	79-80
42	Kallikrein localization in mucous neck cells of the rat stomach antrum (paraffin sections)	82-83
43	Control section for Figure 42	82-83
44	Kallikrein localization in mucous cells and secretions of the rat stomach antrum (semi-thin sections)	85-86
45	Control section for Figure 44	85-86

46	Kallikrein localization in apical regions of mucous cells of the rat stomach antrum (without treatment)	87-88
47	Kallikrein localization in apical regions of mucous cells of the rat stomach antrum (EAI treatment)	87-88
48	Kallikrein localization in apical regions of mucous cells of the rat stomach antrum (PVPP treatment)	87-88
49	Kallikrein localization in submucosal cells of cat respiratory tissue	89-90
50	Control section for Figure 49	89-90
51	Kallikrein localization in secretory epithelial cells of the guinea-pig coagulating gland	92-93
52	Control section for Figure 51	92-93
53	Kallikrein localization in secretory epithelial cells of the guinea-pig coagulating gland (freeze-dried)	94-95
54	Control section for Figure 53	94-95
55	Kallikrein localization in secretory epithelial cells of the guinea-pig coagulating gland (PVPP treatment)	96-97
56	Control section for Figure 55	96-97

57	Kallikrein localization in apical cytoplasm and secretions of the guinea-pig prostate gland	99-100
58	Control section for Figure 57	99-100
59	NGF localization in apical cytoplasm and secretions of the guinea-pig prostate gland	101-102
60	Control section for Figure 59	101-102

LIST OF ABBREVIATIONS AND SYMBOLS

ABC	Avidin-biotin complex
α	Denotes antiserum
α-	Denotes subunit of NGF
β-	Denotes subunit of NGF
γ-	Denotes subunit of NGF
BG	Biotinylated goat anti-rabbit immunoglobulin
BSA	Bovine serum albumin
CDI	1 ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl
CGK	Guinea-pig coagulating gland kallikrein
CSK	Cat salivary kallikrein
DAB	3,3' diaminobenzidine tetrahydrochloride
DMS	Dimethylsuberimidate
EAI	Ethyl acidimidate
EDTA	Ethylene diaminetetraacetate
EGF	Epidermal growth factor
EPTD	Edwards-Pearse tissue drier

FAB	Goat serum immunized against rabbit immunoglobulin G Fab fragments
GMA	Glycol methacrylate
GPCG	Guinea-pig coagulating gland
GPPG	Guinea-pig prostate gland
GPSK	Guinea-pig salivary kallikrein
GTA	Glutaraldehyde
HE	Hematoxylin and eosin
HSK	Human salivary kallikrein
ml	Milliliter, 10^{-3} liter
mm	Millimeter, 10^{-3} meter
mOsm	Milliosmol
μl	Microliter, 10^{-6} liter
μm	Micrometer, 10^{-6} meter
NGF	Nerve growth factor
nm	Nanometer, 10^{-9} meter
Osmium	Osmium tetroxide
PAG	Protein A-colloidal gold complex

PAP	Rabbit peroxidase-antiperoxidase
PAS	Periodic acid-Schiff reagent
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLP	Periodate-lysine-paraformaldehyde
PVPP	Polyvinylpolypyrrolidone
RIK	Rat intestinal kallikrein
RPK	Rat pancreatic kallikrein
SDS	Sodium dodecylsulfate
Tris	Trizma base
Triton	Triton X-100 detergent

I. INTRODUCTION

A. Kallikrein-Kinin System

Tissue kallikreins are a group of serine proteases with common chemical properties and limited proteolytic activity. They release an active peptide from a precursor, or substrate, the peptide being bradykinin, or lysyl bradykinin, the latter also being known as kallidin (see Schachter, 1969, 1980). They release lysyl bradykinin from kininogen, a substrate that is present in lymph, plasma, and interstitial fluid and are, therefore, also called kininogenases. They have effects on blood vessels and smooth muscles *in vivo* that are indirect, i.e. due to the release of the pharmacologically active kinin (lysyl bradykinin). Physiologically significant proteolytic actions of these enzymes may, however, sometimes be unrelated to kinin release (Beraldo *et al.*, 1956; Chao *et al.*, 1981).

The kallikrein story begins in 1909 when Abelous and Bardier described a substance from human urine which lowered blood pressure. The presence of this hypotensive substance in urine was confirmed and it was found to be nondialyzable (Frey and Kraut, 1926). Thought to originate from the pancreas, it was named kallikrein (Greek, "kallikreas" = pancreas) by Kraut *et al.* (1930). The pancreas was later discounted as a major source of the urinary agent since kallikrein levels in dog urine remained high following pancreatectomy or pancreatic vessel occlusion (Beraldo *et al.*, 1956).

Plasma kallikrein differs from the tissue varieties on many grounds. It has a higher molecular weight, resembles trypsin in its substrate requirements, the latter being less specific, and releases bradykinin instead of lys-bradykinin (Colman and Bagdasarian, 1976). The plasma kallikrein-kinin system has been implicated in various pathological states such as inflammation and shock (Colman and Wong, 1979).

The splitting of the arginine-arginine bond by these enzymes indicates that they may be involved in processing prohormones, proenkephalin being a good example (Prado *et al.*, 1983). Kallikrein has also been reported to activate proinsulin (ole-Moi Yoi *et al.*, 1979b) and prorenin

(Sealey *et al.*, 1978).

Measurement of kallikreins is commonly performed chemically by the hydrolysis of various synthetic esters. Bioassay is also performed by recording hypotensive activity of the substance on the dog blood pressure or by measuring the substance's ability to release kinin from a substrate. Aprotinin (or Kunitz's trypsin inhibitor) inhibits tissue kallikrein, which results in prevention of kinin production.

Localization techniques are widely used and allow proteins to be identified and characterized at their sites of synthesis, storage and secretion. Several immunocytochemical techniques have been used to localize kallikrein in various tissues. Frequently, however, immunolocalizations by different authors produced different results. The purpose of this thesis is to immunolocalize tissue kallikrein in various tissues. The diverse cellular and subcellular locations of these enzymes indicate that they have different functions in different organs despite having similar chemical and pharmacological properties (Schachter, 1980).

In this thesis, several related kallikreins are localized immunocytochemically in various gastrointestinal and male reproductive tissues. Freeze drying and chemical fixation techniques are used successfully to immunocytochemically localize tissue kallikrein in the submandibular gland of the cat and guinea-pig where it is in apical regions of striated duct cells. It is also localized in goblet (mucous) cells of the colon and mucous cells of the stomach of the cat and rat. Ultrastructurally, it is localized in the apical secretory granules of striated duct cells in the cat and guinea-pig. Furthermore, kallikrein is immunolocalized in surface epithelial cells of the guinea-pig coagulating and prostate glands; also in submucosa glands of the cat trachea. Nerve growth factor is also immunolocalized in surface epithelial cells in the guinea-pig prostate gland.

1. Salivary gland

A vasodilator substance was found in saliva some fifty years ago (Secker, 1934 a,b) with vascular effects unlike acetylcholine or adrenaline (Gibbs, 1935; Larson, 1935). Feldberg

and Guimarais (1935) also concluded that this vasodepressor substance was not acetylcholine, choline, histamine or any other known depressor substance. They also observed that sympathetic saliva had more depressor activity than parasympathetic saliva and that this activity decreased in successive saliva samples. A vasodepressor substance resembling kallikrein of pancreas and urine was found in salivary glands of several species (Werle and von Roden, 1936). It was also in saliva, presumably in the same form as in the gland. It would seem that all of the above vasodilator substances were, in fact, "salivary kallikrein".

Highest concentrations of kallikrein are in the submandibular salivary glands (Werle and von Roden, 1936) with only low concentrations in parotid and sublingual glands and the rat submandibular gland has the highest concentration of any tissue. Salivary kallikrein was isolated from saliva (Fujimoto *et al.*, 1973) and from submandibular glands of man (Fujimoto *et al.*, 1973; Hare and Verpoorte, 1982), cat (Moriwaki *et al.*, 1976; Fukuoka *et al.*, 1979; Fujimori *et al.*, 1985a), mouse (Porcelli *et al.*, 1976), rat (Brandtzaeg *et al.*, 1976) and guinea-pig (Fiedler *et al.*, 1982). In the rat, submandibular gland kallikrein occurs as multiple isoenzymes (Brandtzaeg *et al.*, 1976) as is also the case in the cat (Fukuoka *et al.*, 1979).

Kallikrein is not present in submandibular glands at birth (Werle *et al.*, 1960), and it is synthesized in the adult rat submandibular gland at a slow rate (Nustad and Vaaje, 1975). Kallikrein content of saliva of older people was found to be five times higher than that of teenagers (Sallay and Nador, 1950).

Kallikrein secretion into saliva is mediated primarily by sympathetic nerve stimulation and probably involves alpha adrenergic receptors (Beilenson *et al.*, 1968; Barton *et al.*, 1975; Ørstavik and Gautvik, 1977; Schachter *et al.*, 1977; Ørstavik, 1978b; Simson *et al.*, 1979; Izumi and Aoki, 1981; Izumi, 1984). Parasympathetic denervation caused the kallikrein content of submandibular glands to fall (Emmelin and Henriksson, 1953), and this reduction in content correlated with a reduction in the size of demilune cells. Kallikrein was therefore originally thought to be present in these cells. Chronic duct ligation also caused kallikrein concentrations in submandibular glands to fall in rabbits (Mattioli and Mattioli, 1947a,b), rats, mice, dogs

(Werle, 1960; Werle and Trautschold, 1963) and cats (Beilenson *et al.*, 1968). Kallikrein was thought to be stored in acinar granules of the guinea-pig submandibular gland (Bhoola and Ogle, 1966). Based on fractionation of gland extracts, the enzyme was absent from mitochondria and lysosomes (Bhoola and Dorey, 1969). Bhoola and Heap (1970) used electron microscopy to compare isolated granules to fractions with kallikrein activity. Based on size and shape, they concluded that granules with the highest activity resembled those from acinar cells and the suggestion was therefore made again that kallikrein was stored there. This conclusion was extended to the cat, dog and rabbit (Bhoola, 1969). Other morphological data also seemed to support a connection between kallikrein release and acinar cell secretion in gland slices (Albano *et al.*, 1976).

A ductal localization of kallikrein was, however, later suggested by morphological studies after sympathetic nerve stimulation and correlations of the concentration of ductal granules with kallikrein content of the gland. In cats, sympathetic stimulation depleted striated duct cells of secretory granules, and depleted the kallikrein content of the gland, but acinar cells were left intact (Barton *et al.*, 1975; Garrett and Kidd, 1975; Schachter *et al.*, 1977). Similar results were reported in guinea-pigs and dogs (Uddin and Tyler, 1978, 1981; Schachter *et al.*, 1980). Parasympathetic nerve stimulation in the cat resulted in loss of acinar secretory granules but no reduction in kallikrein content (Barton *et al.*, 1975; Garrett and Kidd, 1975). Secretory granules from demilune cells were depleted after either sympathetic or parasympathetic stimulation, suggesting that these cells were unlikely sources of kallikrein (Uddin and Tyler, 1978).

Immunocytochemistry clearly established that kallikrein was localized in granular convoluted tubule and striated duct cells in the submandibular glands of rat (Ørstavik *et al.*, 1975; Brandtzaeg *et al.*, 1976; Ørstavik, 1978a; Ørstavik and Glenner; 1978, Simson *et al.*, 1979; Ørstavik *et al.*, 1982a), mouse (Ekfors and Hopsu-Havu, 1971; Simson *et al.*, 1978, 1979), cat (Hojima *et al.*, 1977a; Maranda *et al.*, 1978; Schachter *et al.*, 1980), pig (Dietl *et al.*, 1978), guinea-pig (Schachter *et al.*, 1978, 1980), dog and man (Ørstavik *et al.*, 1980a; Schachter *et al.*,

1980). In sublingual glands of the rat, kallikrein was also localized in the striated duct cells (Ørstavik *et al.*, 1975; Simson *et al*, 1983) and in the luminal rim of striated and larger duct cells (Ørstavik *et al.*, 1975). In parotid and sublingual glands of the rat, kallikrein was again localized in ductal segments (Ørstavik 1978a; Simson *et al.*, 1979; Ørstavik *et al.*, 1982a). In human parotid glands, kallikrein was localized luminally in striated duct cells and to a lesser degree in the larger ducts (Kimura and Moriya, 1984a), and some immunostaining was also reported extracellularly in duct and acinar basement membrane regions.

Enzyme histochemical techniques, using the substrate, val-leu-arg-methoxy-2-naphthylamide, supported the ductal localization of kallikrein. Active enzyme was localized periluminally in submandibular gland granular tubules and striated duct cells in the rat, mouse, hamster, guinea-pig, cat, dog (Garrett *et.al.*, 1982) and man (Garrett *et al.*, 1984). An active enzyme, resembling kallikrein, was also demonstrated in salivary gland mast cells by this enzyme histochemical method. Submandibular, parotid and sublingual glands of monkeys (Arnold, 1984) and other mammals (Arnold, 1983) had kallikrein-like activity by this method also in apical regions of striated duct cells. Active enzyme was also demonstrated histochemically in luminal regions of striated duct cells of the human parotid gland (Kimura and Moriya, 1984a).

Kallikrein was finally localized immunocytochemically by electron microscopy in secretory granules of striated duct cells in cat submandibular glands (Schachter *et al.*, 1983c) (see Results). In rat submandibular glands, the enzyme was similarly localized in secretory granules of granular convoluted tubule and striated duct cells (Simson *et al.*, 1983). Apical membrane and basal lysosomal staining were also reported. Ultrastructural enzyme histochemistry also localized kallikrein-like activity in apical secretory granules in striated duct cells of monkey submandibular glands (Arnold, 1985).

Salivary gland kallikrein may be under endocrine control in some cases. Although submandibular glands of rats and mice failed to show a sexual difference and testosterone treatment did not affect kallikrein levels (Bhoola *et al.*, 1973) Van Leeuwen *et al.* (1984) found

that testosterone increased kallikrein levels in rat submandibular glands. In the hamster, which shows an estrogen-linked biochemical dimorphism of the submandibular glands, female glands contain more kallikrein than male glands (Bhoola *et al.*, 1973), but human saliva kallikrein concentration is probably not estrogen dependent since it did not vary during the menstrual cycle (Bhoola *et al.*, 1978).

2. Pancreas

The pancreas was found to contain large amounts of kallikrein (Kraut *et al.*, 1930). The enzyme exists in the pancreas as a precursor (prokallikrein), and also in the pancreatic secretion (Amouric and Figarella, 1979) and in fresh organ extracts (Hojima *et al.*, 1977b). It is secreted into the duodenum and activated by trypsin and enterokinase (Werle and Semm, 1955). Purification of pancreas kallikrein was achieved in the dog (Hojima *et al.*, 1977b), hog (Fiedler and Gebhard, 1980), rat (Fiedler *et al.*, 1977) cat (Fujimori *et al.*, 1985b) and man (Moriya *et al.*, 1963; ole-Moi Yoi *et al.*, 1979a). Purified enzymes usually existed in multiple forms.

Kallikrein was found to be synthesized as well as secreted by the pancreas (Frey *et al.*, 1968), where the enzyme was suggested to have an exocrine function. Support for this idea came from observations that it was secreted in parallel with trypsin and lipase and because its concentration fell like other digestive enzymes after pancreatic duct ligation (Forell, 1960).

It was hypothesized that kallikrein originated from acinar cells because of the above observations and because its concentration was not affected by treatment which destroyed β-cells of the islets of Langerhans (Forell, 1960). Based on subcellular fractionation, kallikrein activity was detected within secretory granules (Bhoola and Dorey, 1971), and by immunocytochemistry in apical regions of acinar cells in the pancreas of pig (Dietl *et al.*, 1978), rat (Proud *et al.*, 1977; Ørstavik and Glenner, 1978; Ørstavik *et al.*, 1980a, 1981a) and man (Ørstavik *et al.*, 1980a, 1981a). Ultrastructural immunolocalization demonstrated kallikrein in different cellular compartments of rat pancreatic acinar cells (Bendayan and Ørstavik, 1982). It was thus

localized in rough endoplasmic reticulum, Golgi body, condensing vacuoles and secretion granules which supports the view that it is synthesized and secreted by acinar cells. In accord with the above, kallikrein was localized ultrastructurally in porcine pancreatic acinar cells, secretory granules and the Golgi body (Lechene de la Porte *et al.*, 1981).

By radioimmunoassay and bioassay, kallikrein was present in isolated acinar cells, but not in isolated islet cells (Chao *et al.*, 1980). To further resolve localization in different cell types, enzyme concentration was measured following either β-cell destruction by streptozotocin or acinar atrophy by duct occlusion (Ørstavik *et al.*, 1981b). Kallikrein content was decreased with acinar atrophy only, which again indicates that these cells are the site of storage in this organ.

The situation has recently been rendered more complex by ole-Moy Yoi *et al.* (1979a), who, using human antisera to urinary kallikrein, immunolocalized kallikrein in β-cells of human pancreatic islets. Furthermore, by varying experimental conditions, kallikrein-like antigenicity was localized in either islet β-cells or acinar cells (Pinkus *et al.*, 1983). Untreated tissue sections immunostained exclusively in acinar cells but when enzyme-digestion methods were used kallikrein was detected in β-cells. The question of the presence of kallikrein in islet β-cells requires further study.

3. Colon

Hypotensive activity due to kallikrein was first reported in extracts of the colon of several mammals (Werle, 1960). Trypsin-activated kallikrein was found in the colon of man, dog, cat, rat, pig and cow (Frankish and Zeitlin, 1980) and also in colonic mucosa of rat and rabbit (Amundsen and Nustad, 1965). Rat colon extracts also contained kallikrein (Zeitlin, 1970). In the human (Zeitlin and Smith, 1973) and cat colon (Fasth *et al.*, 1978), kallikrein was found mainly in the mucosal layer.

Prokallikrein, activatable by trypsin was reported to be in the colon wall of human, monkey and dog tissues (Seki *et al.*, 1972). On enzymatic and chemical grounds this enzyme

was earlier thought to resemble the plasma kallikreins, but purified human (Zimmermann *et al.*, 1979), cat and rat colon kallikrein (Frankish and Zeitlin, 1980; Fujimori *et al.*, 1985a) have since been consistently found to resemble tissue kallikreins on enzymatic, immunological and chemical grounds.

Kallikrein was localized immunocytochemically in mucous or goblet cells of human, rat and cat colon (Schachter *et al.*, 1983a) (see Results). By enzyme histochemistry, kallikrein-like activity was also detected in mucous cells of the cat and rat colon (Schachter *et al.*, unpublished observations) and also in gastrointestinal mast cells.

The above studies strongly indicate that colon kallikrein is of the tissue variety and is present in goblet cells.

4. Intestine

Kallikrein, activated by trypsin, was detected in the small intestine of many mammals, viz., man, dog, cat, rat, pig and cow tissues (Werle, 1960; Werle and Vogel, 1960). Intestinal mucosal cells of rats and rabbits (Amundsen and Nustad, 1965), extracts of rabbit small intestine (Burger *et al.*, 1968) and extracts of rat intestinal tissues also displayed enzyme activity (Zeitlin, 1970; Frankish & Zeitlin, 1980).

Kallikrein in rat intestinal mucosa was present as an inactive precursor (Seki *et al.*, 1972; Zeitlin *et al.*, 1976), activatable by autolytic processes or by incubation with trypsin (Werle, 1960; Zeitlin, 1971). It behaved enzymatically like a tissue kallikrein, rather than like a plasma kallikrein (Zeitlin 1971) and its molecular weight also indicated that it was similar to other tissue kallikreins (Zeitlin *et al.*, 1976). Kallikrein was localized immuno- and enzyme-histochemically in mucous cells of rat and cat small intestine (Schachter *et al.*, unpublished observations).

3. Stomach

Kallikrein-like activity was found in extracts of rat and rabbit stomach (Amundsen and Nustad, 1965; Zeitlin, 1970; Frankish and Zeitlin, 1980). Activity was detected in rat stomach tissue with an unusually low pH optimum of 5 (Kobayashi *et al.*, 1979); based on enzyme inhibition profiles, this enzyme resembled cathepsin D (Kobayashi and Ohata, 1981). A kallikrein was also purified from rat stomach similar to other tissue kallikreins (Uchida *et al.*, 1980) with a pH optimum of 11. Similarly, a human stomach kallikrein was purified and found to be similar to tissue kallikreins (Uetsuji *et al.*, 1982). A kallikrein-like enzyme was also localized in the mucous cells of cat stomach (Schachter *et al.*, unpublished observations) (see Results).

6. Kidney (Urinary)

A hypotensive substance detected in human urine by Frey and his colleagues (Frey and Kraut, 1926) was first described as a "Kreislaufhormon" (circulating hormone). This hormone, of uncertain origin, was later named kallikrein (Greek, *kallikreas* = pancreas) since it was thought to originate in the pancreas (Frey *et al.*, 1968).

Purified human urinary kallikrein resembled other tissue kallikreins (Moriya *et al.*, 1963; ole-Moi Yoi *et al.*, 1977, 1978, 1979b). Various other kidney and urinary kallikreins have been purified and found to resemble tissue kallikreins from the same species (Nustad, 1970a; Nustad and Pierce, 1974; Moriwaki *et al.*, 1976; Porcelli *et al.*, 1976; Fujimori *et al.*, 1985b).

Kallikrein activity was first demonstrated in rat kidney homogenates and its activity was shown to be enhanced by trypsin treatment (Werle and Vogel, 1961). The suggestion was therefore made that the enzyme first isolated by Kraut *et al.* (1930) was a prohormone. The fact that the molecular weight of renal enzyme was larger than the urinary enzyme suggested to Moriwaki *et al.* (1976) that the urinary kallikrein might be a degradation product of renal kallikrein.

Subcellular localization of kallikrein within the kidney was determined on the basis of biochemical fractionation. Kallikrein activity was detected in the microsomal fraction (Nustad

and Rubin, 1970), in the endoplasmic reticulum (Nustad, 1970b; Ward *et al.*, 1976a), in the plasma membrane (Nustad, 1970b; Ward *et al.*, 1975, 1976a, b), and in lysosomes of kidney cells (de Carvalho and Diniz, 1966; Baggio *et al.*, 1975; Heidrich and Geiger, 1980).

Kallikrein was found to be synthesized by the renal cortex (Nustad, 1970b) and suspensions of renal cortical cells (Kaizu and Margolius, 1975), and dog renal homogenates had the highest levels of such activity in extracts of cortex (Scicli *et al.*, 1976b). Release of kallikrein from rat kidney occurred into the venous effluent and urine (Roblero *et al.*, 1976).

Since the time that Werle and Vogel (1960) showed a reduced urinary excretion of kallikrein following pharmacological destruction of tubule cells, most evidence suggests the presence of kallikrein in the distal nephron tubules. Extracellular secretion of urinary kallikrein from suspended renal cortical cells was increased by aldosterone and decreased by spironolactone, an aldosterone antagonist in distal tubules (Kaizu and Margolius, 1975). Secretion of kallikrein occurred in the distal nephron based on stop-flow analysis of dog kidney (Scicli *et al.*, 1976a).

The proximal convoluted tubule was also reported to contain kallikrein activity (Nustad, 1970b). Reabsorption droplets in proximal tubule cells were immunocytochemically stained (Simson *et al.*, 1979; Pinkus *et al.*, 1981), which suggested that tissue kallikrein was reabsorbed from tubule fluid (Kimura and Moriya, 1984b). By enzyme histochemistry, active rat kidney kallikrein was localized in tubule cytoplasm of the outer medulla and deep cortex, where proximal tubules, ascending limb of Henlé and collecting ducts reside (Kimura *et al.*, 1982). In the human kidney, Kimura and Moriya (1984b) unexpectedly observed kallikrein-like enzyme activity exclusively in proximal tubule cells. Since kallikrein immune staining did not occur in these or other tubule cells, it was suggested that the enzyme activity was due to non-kallikrein esterases. These authors who found kallikrein immune staining in interstitium and basement membrane regions, viewed renal and urinary kallikreins as derived from circulating tissue kallikrein.

In microdissected rabbit kidney, active and inactive kallikreins were detected in connecting tubule cells (Tomita *et al.*, 1981; Omata *et al.*, 1982; Proud *et al.*, 1983; Marchetti *et al.*, 1984). Using immunocytochemistry, kallikrein secretion was indicated by its localization in apical regions of distal tubule cells and in the tubule lumen of rat (Ørstavik *et al.*, 1976; Simson *et al.*, 1979; Ørstavik and Inagami, 1982), mouse (Simson *et al.*, 1979), and human kidney (Pinkus *et al.*, 1981). Kimura and Moriya (1984b) postulated that kallikrein secretion in the distal tubule may not necessarily indicate *de novo* synthesis in tubule cells, where it is secreted into the urine. Kallikrein antigenicity in distal tubule cells may therefore represent tissue enzyme from other sources. Figueroa *et al.* (1984) localized kallikrein immunocytochemically in the cytosol of apical and perinuclear areas of distal connecting tubule cells. The antigenicity pattern within organelles suggested that kallikrein synthesis occurred in this cell type. Basal infoldings of connecting tubule cells also stained, which may explain the appearance of kallikreins in isolated kidney venous effluent (Roblero *et al.*, 1976; Vio *et al.*, 1981, 1982, 1983).

Although the physiological significance is not completely understood, Margolius *et al.* (1972, 1974) observed that urinary kallikrein excretion is abnormal in various hypertensive animal models and in human hypertensive disease. Low sodium diet or high levels of sodium-retaining steroid hormones also increased urinary kallikrein excretion in rat and man (Geller *et al.*, 1972; Margolius *et al.*, 1974). Further observations have been made indicating that kallikrein is under hormonal control in the kidney but the physiological significance of this fact remains obscure (Geller *et al.*, 1972; Margolius *et al.*, 1974).

Kallikrein activity in the nephron tubule was shown to be under corticosteroid hormone control. Urinary kallikrein secretion was stimulated by aldosterone or deoxycorticosterone (Geller *et al.*, 1972; Margolius *et al.*, 1974) and kallikrein activity in the rabbit connecting tubule decreased after adrenalectomy (Marchetti *et al.*, 1984). Chronic deoxycorticosterone treatment also increased activity of kallikrein in the connecting tubule, though not its synthesis (Marchetti *et al.*, 1984).

Possible physiological roles of renal kallikreins were also suggested to be related to the activation of prorenin by kallikrein (Sealey *et al.*, 1978). Though prorenin cleavage resulted in activation, some structural alteration of prorenin was required before activation could occur (Hsueh *et al.*, 1980).

7. Respiratory Tract

Human bronchial secretion has been reported to contain kallikrein (Havez *et al.*, 1966a,b). It was detected in normal "respiratory sputum" and found to resemble tissue kallikreins on enzymatic and immunological grounds. The problem is whether the sputum contained salivary kallikrein, but the authors do not discuss this obvious problem.

8. Male Sex Glands

An arginine esterase was described in the different lobes of rodent prostate glands (Gotterer *et al.*, 1956). The guinea-pig coagulating gland was shown to secrete "toxic" substances (Freund and Thompson, 1957) and in further studies this factor was characterized as a glycoprotein with arginine esterase activity that increased vascular permeability (Freund *et al.*, 1958). Bhoola *et al.* (1962) and Moriwaki and Schachter (1971) found a kinin-releasing "kallikrein" (coagulating gland kininogenase, CGK) responsible for hypotensive, permeability-enhancing and esterolytic actions of coagulating gland extracts. They emphasized the kallikrein-like nature of this kininogenase (CGK). Purified CGK resembled other tissue kallikreins on enzymatic and chemical grounds (Moriwaki *et al.*, 1974). Kinin-producing activity was not detected in coagulating or prostate gland extracts of rat, rabbit, dog or man (Bhoola *et al.*, 1962). CGK is now known to resemble the kallikreins chemically (Fiedler, 1979).

Synthesis of a major protein of canine seminal plasma was found in prostate slices and its concentration was increased by androgens and decreased by castration (Dubé *et al.*, 1983). Isaacs and Shaper (1983) and Chapdelaine *et al.* (1984) determined that this major androgen-dependent protein was a serine arginine esterase similar to tissue kallikreins. This

enzyme was identified as a serine protease of the tissue kallikrein type but its ability to release kinin was apparently not tested (Lazure *et al.*, 1984).

A cytosolic localization of CGK from guinea-pig was found by Barton *et al.* (1973), who demonstrated approximately 90% of the activity was in the soluble fraction of guinea-pig coagulating gland extracts. Immunocytochemically, kallikrein was localized apically in the secretory epithelial cells of the guinea-pig coagulating (GPCG) (Schachter *et al.*, 1978) and prostate glands (GPPG) (see Results).

9. Brain

Kallikrein-like activity was first described in nervous tissue by Werle and Vogel (1961). Rabbit and rat brain homogenates (Shikimi *et al.*, 1972) also had kallikrein-like activity. Highest concentrations were in the cerebral cortex (Shikimi *et al.*, 1972) where kallikrein activity was also reported to be inhibited by aprotinin. Scicli *et al.* (1984) found kallikrein in many regions of the brain, including the hypothalamus.

Simson *et al.* (1984) immunolocalized tissue kallikrein in rat brain. Although staining did not occur in specific cell types, it was found in hypothalmus, ventricular ependymal cells, and cell bodies of certain nuclei. The kallikrein-kinin system was therefore postulated to participate in nervous and neuroendocrine functions (Scicli *et al.*, 1984).

10. Other Tissues

A kallikrein-like enzyme was isolated from porcine pituitary glands and found to resemble other tissue kallikreins (Polivka *et al.*, 1982). Powers and Nasjletti (1983) also reported kallikrein-like activity in the rat neuro-intermediate lobe where it may be involved in pro-opiomelanocortin processing. Synthesis of kallikrein in the anterior lobe was suggested by the finding of kallikrein mRNA in normal and hypersecretory rat pituitaries (Fuller *et al.*, 1985). Powers *et al.* (1984) suggested an involvement with hypertension since lower levels of tissue kallikrein activity were found in genetically hypertensive rats. A possible role in hormone

processing was implied by the observation that kallikrein-like activity of the female rat anterior pituitary was fifteen times higher than that of the male (Powers and Nasjletti, 1984).

Kinin-releasing proteases were also isolated from blood vessels (Nolly and Lama, 1982; Nolly *et al.*, 1983), rat cardiac tissue (Britos and Nolly, 1981) and rat spleen (Chao *et al.*, 1984b). With allowances for minor variations, these enzymes can be included in the tissue kallikrein family. Tissue kallikrein, activatable by trypsin (Chao *et al.*, 1984a), was found in intact human erythrocytes and ghosts. Its possible relation to erythrocyte maturation or to membrane transport remains to be established.

Rat thyroid gland also contains a kinin-releasing esterase resembling other tissue kallikreins (Uchida *et al.*, 1982). Chemical and physical properties of this enzyme were established but its physiological role is unknown.

B. Nerve Growth Factor

This substance is included because, as described below, it often co-exists in tissues with kallikrein, and is also associated with a kallikrein-like molecule in a macromolecular complex or aggregate.

Nerve growth factor (NGF) is a molecular complex possessing a subunit with some chemical and physical properties which are similar to those of the kallikreins (Thoenen and Barde, 1980; Darling *et al.*, 1983; Calissano *et al.*, 1984). NGF was discovered when a substance was found in sarcomas, snake venoms, and elsewhere, which stimulated growth of chick sympathetic neurons (Bueker, 1948; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini and Booker, 1960).

The richest known source of NGF is the submandibular gland of the male mouse (Levi-Montalcini and Angelleti, 1961). Submandibular glands of the rat, guinea-pig, cow, pig, rabbit and man contain little or no NGF (Harper and Thoenen, 1980). NGF was recently found in prostate glands, though not all prostate glands contain it. Of several animals surveyed, only guinea-pig, rabbit and bull showed NGF activity in prostatic tissue (Harper *et al.*, 1979;

Harper and Thoenen, 1984). Purification of guinea-pig prostatic NGF was achieved by Chapman *et al.* (1981). Bovine seminal plasma NGF was purified and found to resemble mouse submandibular gland NGF in biological and immunological properties (Harper and Thoenen, 1984). Other rich sources of NGF include snake venom (Cohen, 1959), human placenta at term (Goldstein *et al.*, 1983) and submandibular and prostate glands of the house musk shrew (*Suncus murinus*) (Ueyama *et al.*, 1981).

Male mouse submandibular gland NGF was purified and characterized (Varon *et al.*, 1968; Server and Shooter, 1977) and found to be composed of three subunits (α, β, γ), each of which is dimeric (Varon *et al.*, 1968). One or two bound zinc ions further stabilize the complex (Pattison and Dunn, 1975). The α-subunit has not been shown to have enzymatic activity, the complex's biological activity on nerve growth residing with the β-subunit (Varon *et al.*, 1968). The β-subunit promotes survival, differentiation and maintained function of sympathetic nerve cells (Mobley *et al.*, 1977a, b, c) and may also be the chemotactic agent for neurite outgrowth (Campenot, 1977). The γ-subunit is a potent kallikrein-like arginyl esteropeptidase (Green *et al.*, 1969; Moore *et al.*, 1974; Thomas and Bradshaw, 1981). Bothwell (1979) and Fiedler and Fritz (1981) described large sequence homologies between γ-NGF and tissue kallikreins. The activity of γ-NGF is to cleave the β-NGF precursor (pro-β-NGF) to yield β-NGF (Berger and Shooter, 1977). Morris *et al.* (1981) found that the γ-NGF subunit also activates inactive renin.

Synthesis of NGF and differentiation of secretory tubule cells of rodent submandibular glands were shown to be under control of testosterone (Levi-Montalcini and Angeletti, 1961; Goldstein and Burdman, 1965). NGF was reported to be located in the granular convoluted tubules of the rodent submandibular gland (Levi-Montalcini and Angeletti, 1964).

Light microscopy and subcellular immunocytochemical localization indicated an association of NGF with cytoplasm, secretory granules, the basal part of the cell and the nucleus (Levi-Montalcini and Angeletti, 1961; Goldstein and Burdman, 1965; Kumar *et al.*, 1972). These early conclusions were based on inadequate methods, and later light microscopic

and ultrastructural immunocytochemical studies showed NGF localization in apical secretory granules of secretory tubule (or duct) cells and in saliva (Schwab *et al.*, 1976). The African rodent, *Praomys (mastomys) natalensis*, has high levels of NGF in submandibular glands, where it was also immunolocalized in the granules of the convoluted tubules (Aloe *et al.*, 1981). NGF was also immunolocalized in Suzmann cells or other associated dense fibres in the rat iris (Rush, 1984), and in GPPG within epithelial cells and luminal secretions (Shikata, 1984).

II. MATERIALS AND METHODS

A. Animals, Organ Dissection, Tissue Preparation and Extracts

Animals were kept in a stable environment prior to surgery. Animals were then starved for 24 hours and water allowed *ad libitum* for several hours prior to surgery.

Cats of either sex, weighing 2-4 kg, were anaesthetized with intraperitoneal sodium pentobarbital (M.T.C. Pharmaceuticals, 30-35 mg/kg). A midline incision was followed by blunt dissection to reveal submandibular glands on either side of the trachea near the junction of the transverse vein with the external jugular vein. A small area of gland was separated with clamps and removed while blood flow was intact. Tissue was rinsed briefly in saline and minced on a paraffin-covered dish. Mincing usually took place in fixative unless tissues were to be freeze-dried, where mincing took place in saline. Final tissue sizes were typically less than 1 mm^3. Cat trachea was obtained similarly. Pieces of trachea near the bifurcation were removed, rinsed in saline and minced. The stomach was delivered through an incision and small pieces of tissue were obtained from fundus, corpus or antrum regions. Ascending, transverse and descending portions of the colon were also removed. Upon removal, tissues were rinsed in saline and minced. In some cases, only the mucosal layer, which was stripped of muscular layers, was used.

Rats of either sex weighing 300-400 grams were anaesthetized with intraperitoneal sodium pentobarbital (40-50 mg/kg). Gastric and colonic tissues were removed as before. Usually, only stomach antrum mucosa or descending colon mucosa were removed, rinsed in saline and minced.

Male guinea-pigs, weighing 600-1200 grams, were anaesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Animals were prepared as above and submandibular glands and sex glands identified. Pieces were removed, rinsed with saline and minced. Seminal vesicles, coagulating gland, and dorsal and ventral prostate glands were identified. Pieces of paired coagulating glands, consisting of larger, more transparent lobes, and paired dorsal or lateral

prostate glands were removed, rinsed in saline and minced. Pieces were also transferred into fresh fixative or onto prepared surfaces for rapid freezing.

Extracts were prepared for immunodiffusion experiments from various tissues. Tissues were cut into small pieces, mixed in 5 volumes of distilled water and stirred for 4 hours at room temperature. Mixtures were centrifuged for 10 minutes at 10,000 g and pellets were homogenized and resuspended with distilled water. Two more centrifugations followed, with pellets being homogenized and resuspended. Supernatants were collected, pooled and freeze-dried. Powders were resuspended in distilled water, centrifuged and freeze-dried.

B. Fixation of Tissues

1. Chemical Fixation

Fixative solutions were developed for the dual purpose of preserving ultrastructure while retaining antigenicity. Hundreds of combinations were tested for various tissues, with most intense investigation of the cat submandibular gland. Factors which determined a fixative's suitability were concentration of cross-linking reagent, fixative osmolarity and time of fixation. Factors which were less important were type of buffer, fixation temperature, pH (range 7.0-7.5) and addition of $CaCl_2$. A summary of major fixatives follows.

For the cat submandibular gland, most commonly employed cross-linking reagents were aldehydes. Fixatives which used only one type of aldehyde were 1% glutaraldehyde (GTA) (Analychem Corp. Ltd. or J.B. EM Services, Inc.) and 2% or 4% paraformaldehyde (PFA) (Fisher Scientific Ltd.) made from depolymerized powder. Fixatives were made in 0.1 M sodium cacodylate (Analychem Corp. Ltd.) and adjusted to a pH between 7.0 and 7.5 with either NaOH or HCl. Solutions were prepared 1-2 hours before use. Tissues were kept in fixative for 1-2 hours at room temperature. PFA and GTA combinations were often employed. Concentrations of GTA ranged from 0.7%-1.5%, most often held at 1%, while PFA concentrations ranged from 2%-4.5%, often held at 3%. Acrolein (Analychem Corp. Ltd.) was

sometimes added at a concentration of either 0.1% or 1%, but this caused masking of antigenicity. Buffering of fixatives was achieved with either 0.05 M or 0.1 M cacodylate adjusted to a final pH between 7.0-7.5. Sodium phosphate buffers (pH 7.2) were also used. Molarity varied from 0.075 (180 mOsm, measured by freezing point depression with an Osmette precision osmometer) (Precision Systems) - 0.135 M (300 mOsm). Time of fixation for these aldehyde combinations varied from 1-2 hours except for those using acrolein, which was 15 minutes. Often used to determine ultrastructure for a gland was a relatively concentrated combination of aldehydes arrived at by Barton *et al.* (1975). It consisted of a mixture of 2.5% GTA, 4% PFA and 1% acrolein in 0.05 M cacodylate, pH 7.3. Primary fixation was for 2 hours followed by 1 hour post-fixation with cacodylate-buffered 1% osmium tetroxide (osmium) (Analychem Corp. Ltd.), pH 7.3.

Cat submandibular gland fixation was examined using the bifunctional reagent, 1 ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (CDI) (Sigma Chemical Co.) in concentrations of 1% and 10%. CDI was also combined with aldehyde reagents. GTA ranged from 0.5%-1%, while CDI levels were either 2%, 4% or 6%. Also, combinations were used where CDI was 1% or 2%, GTA was 0.5% and PFA was 1%, 2% or 3%. These primary fixatives were used at 4°C for 0.5-4 hours. In some cases, tissues were post-fixed with 1% osmium buffered with 0.05 M cacodylate, pH 7.3, for 15 or 30 minutes at room temperature. Although the buffering system for CDI combinations was occasionally 0.05 M Trizma base (Tris) (Sigma Chemical Co.), 0.1 M sodium phosphate or phosphate-buffered physiological saline, 265 mOsm (Lewis and Knight, 1980), all at pH 7.2, usually a phosphate-buffered saline (PBS)/Tris buffer (Willingham and Yamada, 1979) was used which had a final pH of 7.0 and an osmolarity of 360 mOsm. Osmolarities for solutions containing combinations of CDI, GTA and PFA ranged from 260-1200 mOsm with most suitable osmolarities below 500 mOsm.

Other fixatives were used to compare with the reagents described above. The periodate-lysine-paraformaldehyde (PLP) solution (McLean and Nakane, 1974) was used. It was modified according to Reissig *et al.* (1978) by varying PFA concentrations from 1%-2%,

periodate kept at 0.01 M, lysine at 0.075 M and buffered with either sodium phosphate or cacodylate. The pH was adjusted before use to 7.2 and fixation was for 4 hours at room temperature. Also, 1% acrolein was added to the PLP fixative and used for 15 minutes. Other schemes included Bouin solution (Humason, 1979), 80% ethanol or 1% osmium in Millonig's buffer (Millonig, 1961). Most of these fixatives distorted ultrastructure or masked antigenicity. cat submandibular gland tissue was also perfusion-fixed by injecting 100 ml of fixative into the cannulated carotid artery of an anaesthetized animal. PLP, with or without 3% dextran (Sigma Chemical Co.) or 0.05 M cacodylate-buffered 1% GTA, pH 7.2, was used as perfusant. After 5 minutes, glands were removed and processed in fixative for an additional 4 hours. Although perfusion techniques were successful, they were also cumbersome and results were comparable to immersion fixation.

In preserving guinea-pig submandibular gland ultrastructure, similar fixatives to those used for the cat submandibular gland were developed. Combinations were used where GTA ranged from 0.5%-1.3% and PFA was kept at either 2% or 3%. Acrolein was sometimes added to a level of 0.1%, 1%, 2% or 5% CDI was used in conjunction with either 0.5% GTA or 0.5% GTA and 1% PFA. Post-fixation with 0.1 M cacodylate-buffered 1% osmium, pH 7.1, for 15 minutes was sometimes tried. 10% CDI was also used for 24 hours at 4℃. When CDI was included in the fixative, PBS/Tris buffer was used and the final pH adjusted to between 7.0 and 7.5. 0.05 M or 0.1 M cacodylate was used to buffer these mixtures in the same pH range. The concentrated aldehyde fixative used for the cat submandibular gland was also used for the guinea-pig submandibular gland to examine regular ultrastructure. Other unsuccessful fixatives included cold 95% ethanol or 2% dimethylsuberimidate (DMS) (Sigma Chemical Co.) in 0.15 M Tris, pH 9.5, to which 0.02 M $CaCl_2$ was added (Hassel and Hand, 1974). These last two fixatives were used for 4 and 2 hours, respectively, but caused cell distortion.

Solutions used to fix pieces of cat colon included aldehyde-based mixtures such as 1% GTA-3% PFA for 2 hours, 1% CDI-0.5% GTA for 1 or 2 hours, all in PBS/Tris buffer. 1% CDI was also used alone or in combination with 2% PFA with or without the presence of 0.1%

saponin (Fisher Scientific Co.) made in either PBS/Tris buffer or 0.1 M sodium phosphate, pH 7.4. 4% PFA was used in sodium phosphate buffer, pH 7.4, for 24 hours at 4°C. PLP, cold 95% ethanol or 0.5% osmium in 0.075 M cacodylate, pH 7.0, were also used to fix tissue.

Rat colon pieces were fixed in solutions of 1% CDI in PBS/Tris buffer, pH 7.0, or PLP for 3 hours. Osmium was used at three concentrations: 0.5%, 1% and 2% in 0.1 M cacodylate buffer, pH 7.0.

Fixatives for the cat stomach were 1% CDI, 1% CDI-0.5% GTA or 1% GTA-3% PFA in PBS/Tris buffer, pH 7.3, for 1-2 hours. 1% CDI-2% PFA in 0.1 M phosphate buffer, pH 7.4, with or without 0.1% saponin, was also used for 2 hours. 4% PFA was used in either 0.1 M phosphate buffer for 24 hours at 4°C or 0.1 M cacodylate buffer for 30 minutes followed by post-fixation with 0.7% osmium in 0.1 M cacodylate buffer, pH 7.0, for 2 hours. Three concentrations of osmium, 0.5%, 0.7% and 1%, were used in 0.05 M, 0.075 M or 0.1 M cacodylate buffer, pH 7.0-7.4, for 1-2.5 hours. PLP and cold 95% ethanol were also used.

Rat stomach was fixed in either 1%, 3% or 5% CDI or 1% CDI-2% PFA in PBS/Tris buffer. PLP was used for 3 hours, or 0.5%, 1% and 2% osmium in 0.1 M cacodylate buffer, pH 7.2, were used for 1.5 hours.

Fixatives for cat trachea were PLP, 0.5% osmium in 0.05 M cacodylate buffer, pH 7.2, or a combination of 1% CDI and either 2% or 3% PFA. The effect of adding 0.1% saponin was also tested in 1% CDI-2% PFA mixtures.

For ultrastructure, guinea-pig male sex glands were fixed in a modified aldehyde solution after Barton *et al.* (1975). GTA was either 2.5% or 3% with 3% PFA in PBS/Tris buffer. Post-fixation was with 1% osmium for 1 hour. Other combinations included 0.5% or 1% GTA with 2% or 3% PFA in 0.05 M cacodylate buffer. CDI concentrations of 1% or 2% were used with or without 0.5% GTA, and PFA levels ranged from 1%-3%. In some cases, a 15 minute post-fixation step with 1% osmium in 0.05 M cacodylate buffer was added. Osmium as a primary fixative was also used in concentrations of 0.5%, 1% or 2%. Other fixatives included cold 95% ethanol or 2% DMS as described for guinea-pig submandibular gland.

Fixatives that were specifically tested on GPCG pieces were 0.2% or 0.5% GTA with either 1% or 2% PFA. 2%, 3% or 6% PFA was also used. Fixatives used for male sex glands which were used for pre-embedding immunostaining systems are described in that section.

Following fixation, all tissues were washed for at least 1 hour in several changes of fixative buffer. If post-fixation was involved, it followed a buffer wash and after post-fixation, a buffer wash corresponding to this step was used. Washes were followed by dehydration in ascending ethanol concentrations (30%, 50%, 70%, 90%, 95% and 100% twice). If acrolein was part of the fixative, methanol instead of ethanol was used. Xylene was used as intermediate solvent for tissues to be embedded in paraffin (Paraplast Plus) (Fisher Scientific Co.). Propylene oxide was used as an intermediate solvent for tissues to be embedded in epoxy resin. Tissues processed into glycol methacrylate (GMA) (Analychem Corp. Ltd.) were dehydrated without alcohol.

To enhance antigenicity by protecting tissue with chemical treatments, a modified method of Kuhlmann and Krischan (1981) was used. Cat and rat stomach, GPCG and cat trachea were treated with ethyl acidimidate HCl (EAI)(Sigma Chemical Co.) as follows. After fixation, tissues were washed in several buffer changes for at least 2 hours and then treated with 0.2 M EAI dissolved in 0.2 M K_2HPO_4, pH 7.3, for 2 hours at 4°C. A 1 hour buffer wash following treatment was followed by routine dehydration. Rat stomach and GPCG were additionally used to examine polyvinylpolypyrrolidone (PVPP) (Sigma Chemical Co.) treatment to enhance antigenicity. After fixation and buffer wash, tissues were treated with 10% PVPP in buffer for 2 hours. A routine dehydration schedule followed, with all concentrations of ethanol containing 10% PVPP. Tissues were then transferred through two changes of propylene oxide before embedding.

2. Freeze-Drying

Tissue pieces were rapidly frozen either by copper block or cryogenic fluid methods. Cryogenic fluids such as 2-methylbutane (Eastman Kodak Co.) or freon (Analychem Corp.

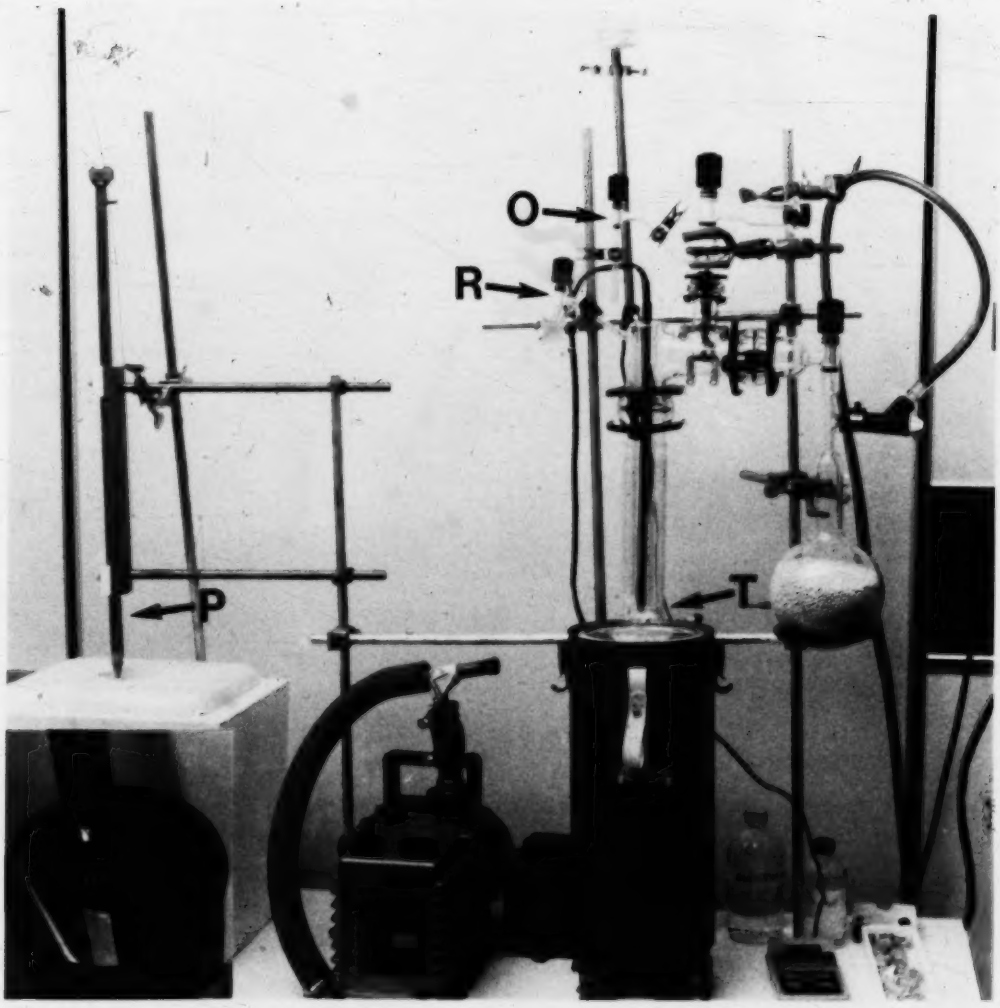
Ltd.) were cooled to their melting point with liquid nitrogen. Tissue pieces were frozen by immersion in cryogenic fluid and then freeze-dried in an Edwards-Pearse tissue drier (EPTD) (Edwards High Vacuum) at -60°C for 24-48 hours. No vapor fixation step was used before embedding in vacuum-treated epoxy resin. This method was used to preserve cat submandibular gland, guinea-pig submandibular gland, GPCG and GPPG tissues.

The copper block method of freezing (Figure 1) (Coulter and Terracio, 1977) was used for cat submandibular gland, guinea-pig submandibular gland, cat colon, rat colon, cat stomach, rat stomach, cat trachea, GPCG and GPPG tissues. Pieces of tissue were placed on thin-gauge aluminum foil on cork stoppers and blotted of excess moisture. Stopper bases were attached to the brass plunger which rapidly delivered tissues onto the polished surface of copper, maintained at liquid nitrogen temperature. Pieces were kept in contact with the block for at least 20 seconds and then delivered into the freeze-drier. Some tissues were processed in the EPTD as above, and others were freeze-dried in an all-glass freeze-drier (Coulter and Terracio, 1978). This device was cooled with liquid nitrogen to keep tissues below -137°C while being dried and an E2M2 doublestage vacuum pump (Edwards High Vacuum) kept pressure below 10^{-3} torr. In some cases, osmium was used for 1-4 hours to vapor-fix tissues. Tissues were embedded into vacuum-treated epoxy resins. Both cryogenic fluids or the copper block method to freeze tissues produced reasonable structure for light microscopy. Ultrastructure, however, was poor and ice crystal artifact was evident.

C. Embedding

Tissues were embedded in paraffin using at least two changes of melted paraffin (melting point 56°C). Araldite and Epon epoxy resins (Analychem Corp. Ltd.) were used according to Hayat (1970). Following the propylene oxide stage of dehydration, a 1:1 ratio of resin:propylene oxide mixture was used on tissues for 24 hours. Tissues were then embedded in capsules filled with resin and polymerized at 60°C for 48 hours.

FIGURE 1. All-glass, low-temperature freeze-drier and copper block freezing device (inset). O, osmium port; P, plunger; R, resin port; T, tissue trough, holds tissue pieces which are cooled by liquid nitrogen (in dewar flask below trough). (After Coulter and Terracio, 1977, 1978).


O
R
P
T

Tissues that were freeze-dried were usually embedded in Spurr low-viscosity embedding medium (Analychem Corp. Ltd.) according to Spurr (1969). Polymerization occurred at 60°C for 24 hours. Other tissues were embedded in water-miscible GMA. According to Leduc and Bernhard (1967), dehydration with water-GMA monomer mixture was followed by overnight GMA polymer impregnation. Polymerization of embedded tissues was with long wavelength UV light for 8-48 hours.

D. Immunocytochemistry

1. Antisera

Antigens, previously purified, had high esterolytic activities and yielded single bands on disc electrophoresis. Cat salivary kallikrein (CSK), guinea-pig salivary kallikrein (GPSK) and human salivary kallikrein (HSK), purified according to Fujimoto *et al.* (1973) and Fukuoka *et al.* (1979), rat pancreatic kallikrein (RPK) (Hojima *et al.*, 1977b), rat intestinal kallikrein (RIK) (Moriwaki *et al.*, 1974, 1980) and guinea-pig coagulating gland kallikrein (CGK) (Moriwaki *et al.*, 1974) were all generous gifts of the late Dr. C. Moriwaki.

Kallikrein antisera were raised in New Zealand rabbits (Schachter *et al.*, 1980) and blood for immune sera was collected from ear veins. Blood from hyperimmunized rabbits was also collected. NGF rabbit antisera were purchased from Collaborative Research Inc. They were raised against 2.5s NGF.

Antisera produced single precipitin lines when reacted with purified antigen or tissue extracts. Although stomach and trachea extracts did not show immune precipitation when reacted with αCSK, double diffusion analysis may not have been sensitive enough to detect small amounts of antigen. Specificity of αCSK was shown (Figure 2) by its ability to form single precipitin lines with cat submandibular gland extracts (peripheral wells 2, 4 and 6). Single precipitin lines were also shown for diffusion of αCSK (center well) and cat colon extracts (peripheral wells 1, 3 and 5). Partial antigenic non-identity between cat submandibular gland

FIGURE 2. Double diffusion analysis of αCSK (center well) with cat colon (well 1, 10 μg; well 3, 20 μg; well 5, 30 μg) and cat submandibular gland (well 2, 10 μg; well 4, 20 μg; well 6, 30 μg) extracts. Note single precipitin line and spur formation between lines from peripheral wells 2 and 3.

1
2
3
4
5
6

and cat colon antigens was disclosed by spur formation between lines from peripheral wells 2 and 3.

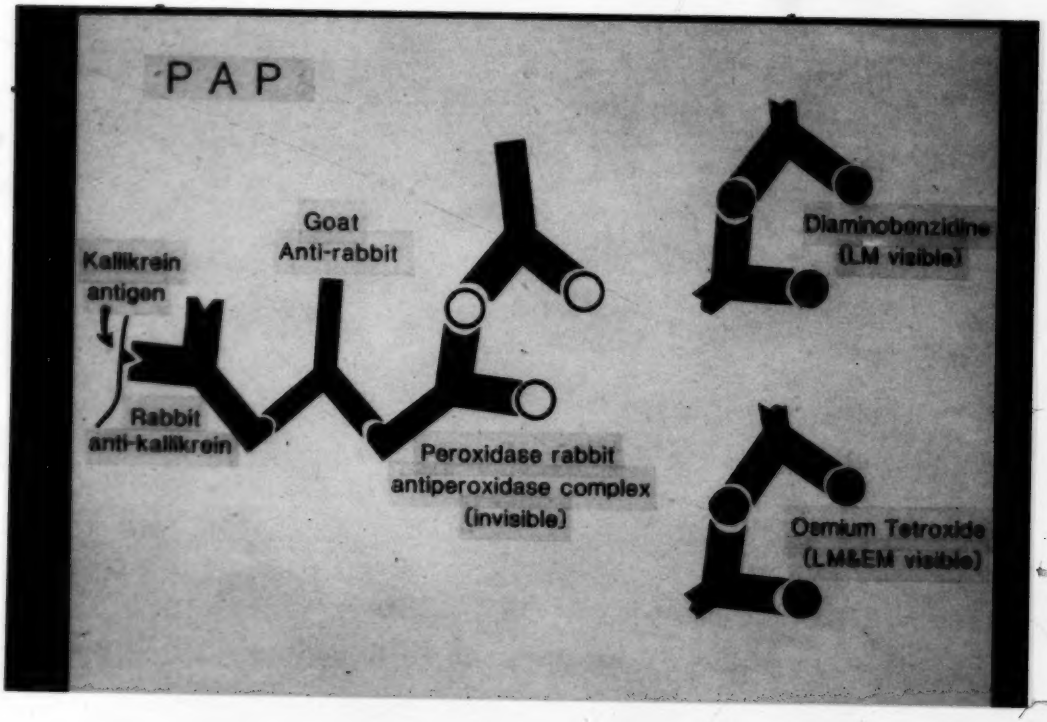
2. Immunostaining systems

a. Peroxidase-antiperoxidase

i. Paraffin sections

Peroxidase-antiperoxidase staining procedures (Sternberger, 1979) (Figure 3) were modified for paraffin-embedded tissues. Seven μm thick sections were cut and mounted on gelatin-coated glass slides. Removal of paraffin with several changes of xylene was followed by rehydration through a descending ethanol concentration gradient (100% twice, 95% twice, 70%) and washing in PBS or 0.05 M Tris buffer, pH 7.6. Excess moisture was removed and slides were put into a humidity chamber. Sections were incubated for 30 minutes at room temperature in normal goat serum (diluted 1:30) (Gibco Laboratories). Unless otherwise stated, all dilutions are in 0.05 M Tris buffer, pH 7.6, and all normal goat sera dilutions are 1:30. They were blotted and covered with either immune or normal rabbit serum (diluted 1:1000-1:5000 with buffer containing 1% normal goat serum, optimally diluted 1:2000). Unless otherwise stated, all primary sera were diluted in buffer with 1% normal goat serum. Humidity chambers were covered and sections were incubated for 24 hours at 4°C. Sections were rinsed with buffer, incubated for 30 minutes with goat serum immunized against rabbit immunoglobulin G Fab fragments (FAB) (Miles Laboratories Inc.) (diluted 1:20-1:80, optimally diluted 1:40), rinsed in buffer, incubated for 30 minutes with rabbit peroxidase-antiperoxidase (PAP) complex (Miles Laboratories Inc., diluted 1:30-1:100, or Cappel Laboratories, diluted 1:50-1:120, optimally diluted 1:100), rinsed with buffer, and finally incubated with a freshly prepared, filtered solutions of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co.) and 0.01% H_2O_2 (J.T. Baker Chemical Co.) in buffer. Reaction was stopped after

FIGURE 3. Summarized PAP method of immunostaining. LM, light microscopically; EM, electron microscopically. (After Sternberger, 1979.)


PAP
Kallikrein antigen
Goat Anti-rabbit
Rabbit anti-kallikrein
Peroxidase rabbit antiperoxidase complex (invisible)
Diaminobenzidine (LM visible)
Osmium Tetroxide (LM&EM visible)

10-15 minutes by rinsing slides in distilled water. Slides were dehydrated in xylene and mounted with Permount (Fisher Scientific Co.). Paraffin-embedded tissues and antisera tested for immunostaining were as follows. The cat submandibular gland was tested with αCSK and αCGK. The cat colon was tested for reactivity with αCSK and αCGK, and the rat stomach was tested with αCSK, αRPK and αRIK.

For routine light microscopic examination of structure, paraffin sections were stained with hematoxylin and eosin (HE) (Humason, 1979) or by the periodic acid-Schiff reagent (PAS) (Fisher Scientific Co.) of Humason (1979).

ii. Semi-thin resin sections

Procedures for semi-thin resin section immunostaining were similar to those described by Schachter *et al.* (1983a). Sections measuring 0.5 μm to 1.5 μm thickness were cut on a Reichert-Jung ultracut (Sargent-Welch Scientific Co.) and dried onto gelatin-coated glass slides on a hot plate at 200°C. Resin was removed by treating slides for 30-90 minutes with 30%-100% saturated ethanolic or methanolic sodium ethoxide (Aldrich Chemical Co.). In some cases, slides were treated with a similar solution made at least 1 week before use by dissolving NaOH pellets in absolute ethanol (Lane and Europa, 1965). Following etching, slides were rinsed twice in absolute ethanol (or methanol), twice in distilled water and immersed in buffer for at least 30 minutes. In some cases to reduce background staining for tissues fixed with osmium, a distilled water rinse-H_2O_2 treatment (5% or 10% for 5-15 minutes) -distilled water rinse sequence was introduced between the earlier distilled water rinses. For the cat colon, detergent treatment was sometimes attempted to enhance penetration of staining reagents into fixed tissue (DeArmond *et al.*, 1981). 2% sodium dodecyl sulfate (SDS) (Terochem Laboratories Ltd.) in PBS with 10^{-3} M ethylene diaminetetraacetate (EDTA) (Sigma Chemical Co.) was used for 10 minutes. Slides were rinsed twice with PBS-EDTA and then Tris buffer. Following etching, sections were incubated in normal goat serum for 15-30 minutes at room temperature. In some experiments, this step was omitted to test normal goat serum's

effectiveness in blocking sites of non-immune or unspecific binding of reagents. They were blotted with Whatman No. 4 filter paper (Fisher Scientific Co.) and covered with either immune or normal rabbit serum (diluted 1:500-1:10,000). Incubations were carried out in humidity chambers for 48-72 hours at 4°C. Sections, at room temperature, were rinsed with buffer, blotted, incubated for 30 minutes in FAB (diluted 1:10-1:80), rinsed with buffer, blotted, incubated for 30 minutes in PAP (diluted 1:30-1:120), rinsed in buffer, blotted and incubated in a freshly prepared, filtered solution of 0.025% DAB and 0.005% H_2O_2 in buffer, until a brown reaction product appeared (usually 5-10 minutes). Slides were rinsed in distilled water, dried at 60°C for at least 1 hour and mounted with Permount.

Semi-thin resin-embedded tissues and antisera tested with the PAP method were prepared as follows. The cat submandibular gland was tested with αCSK and the guinea-pig submandibular gland was tested with αGPSK. The cat colon was tested with αCSK, αRPK, αRIK, αGPSK, αHSK and αCGK and the rat colon was tested with αCSK. Cat stomach tissue was tested with αCSK and αRPK and the rat stomach was examined using αCSK. Cat trachea was tested with αCSK, GPCG was tested with αCGK and GPPG was tested with αCGK, αCSK and αNGF. Cat submandibular gland, cat colon, cat and rat stomach semi-thin sections were also stained by the PAS method, either alone or in conjunction with immunostaining. When both staining methods were used together, PAS staining for carbohydrates followed PAP immunostaining. Sometimes, semi-thin resin sections were stained for regular light microscopic histology, after being etched, with HE and PAS. Usually, they were stained before etching with methylene-blue-azur B (Richardson *et al.*, 1960). Slides were dried and coverslipped as before.

iii. Ultrathin resin sections

Ultrathin immunostaining procedures were modified after Schachter *et al.* (1983a, 1983c). 60 to 80 nm sections were cut on a Reichert-Jung ultracut, expanded with xylene vapor and mounted on 150 or 200 mesh nickel grids, previously coated with Formvar (Ladd Research Industries Ltd.). In some instances, sections were etched with either 1% or 2% saturated

ethanolic sodium ethoxide for 3-10 minutes or 5% or 10% aqueous H_2O_2 for 5-10 minutes. An attempt to reduce background staining was also made by incubating grids in methanol for 5 minutes followed by 0.01% H_2O_2 in methanol for 5 minutes to block endogenous peroxidase activity (Streefkerk, 1972). Grids were rinsed in distilled water, buffer, and incubated with normal goat serum for 5 minutes at room temperature. They were blotted with Whatman filter paper and submerged in drops of immune or normal rabbit serum (diluted 1:500-1:15,000 as before, optimally diluted 1:5000) or in the presence of 0.001%, 0.01% or 0.05% Triton X-100 detergent (triton) (Sigma Chemical Co.) in a humidified chamber after Pelliniemi and Van Noorden (1977). Incubations were carried out for 24-96 hours at 4°C. Staining took place at room temperature as follows. Grids were rinsed with buffer, blotted, floated for 5 minutes on normal goat serum, blotted, incubated for 5 minutes with FAB (diluted 1:10-1:90, optimally diluted 1:30), rinsed with buffer, blotted, floated on normal goat serum for 5 minutes, blotted, incubated for 5 minutes in PAP (diluted 1:40-1:100, optimally diluted 1:60), rinsed with buffer and loaded into a modified grid storage box with holes of 2 mm diameter drilled into each chamber to allow good flow of reagents. In some cases, an e.m. multiple grid staining kit was used (Analychem Corp. Ltd.). Without allowing them to dry completely, up to 20 grids were reacted together with a freshly prepared, filtered solution of 0.0125% DAB and 0.0025% H_2O_2 in buffer. The reagents were magnetically stirred while grids were incubated for 3-5 minutes. Grids were rinsed twice in distilled water, dried and floated on 4% aqueous osmium for 15-30 minutes. They were again rinsed twice in distilled water and allowed to dry. In some cases, this osmium step was omitted.

Ultrathin resin-embedded tissues and antisera tested with the PAP method were as follows. The cat submandibular gland was tested with αCSK and guinea-pig submandibular gland with αGPSK and αCSK. The GPCG and GPPG were examined with αCGK and cat colon tissue was examined with αCSK. Ultrastructure was examined by staining ultrathin sections with a double staining procedure (Pease, 1964). Grids were first processed in 5% uranyl acetate in methanol for 10-60 minutes, rinsed several times in methanol and dried.

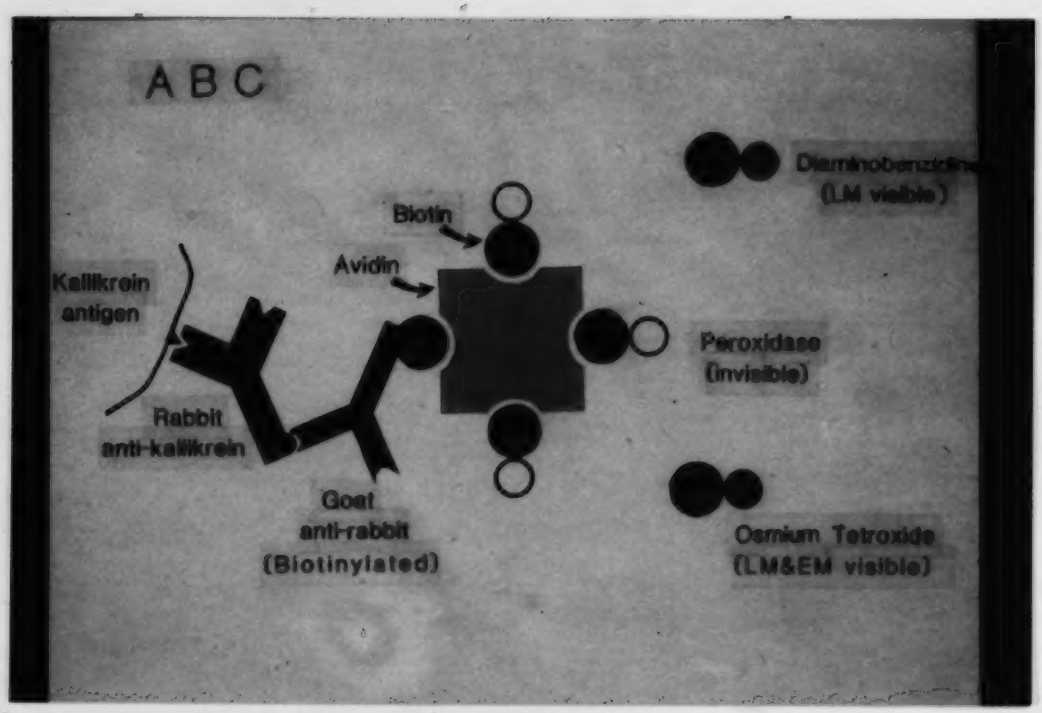
Secondly, grids were floated on drops of freshly prepared lead citrate solution (Reynolds, 1963) for 1-3 minutes, rinsed in distilled water and dried before being observed in the electron microscope.

b. Avidin-biotin complex

i. Semi-thin resin sections

Avidin-biotin-complex staining procedures (Hsu *et al.*, 1981) (Figure 4) were used for semi-thin resin sections. 0.5-1.5 μm sections were cut and dried onto gelatin-coated glass slides. Sections were treated with sodium ethoxide to remove resin and, where appropriate, treated with H_2O_2 as described before. Enhancement of immunostaining by protease digestion was tried on GPCG sections by using 0.1% trypsin (from bovine pancreas) (Sigma Chemical Co.) in 0.05 M Tris buffer containing 0.1% $CaCl_2$, pH 7.6. Incubation took place at 37°C for 10-20 minutes and was followed by rinsing in buffer before proceeding. Following etching, sections were incubated in normal goat serum for 15-30 minutes. They were blotted and covered with either immune or normal rabbit serum (diluted 1:500-1:10,000, optimally diluted 1:5000). Incubations were carried out in humidity chambers for 24-72 hours at 4°C. At room temperature, sections were rinsed with buffer, blotted, incubated for 30-60 minutes in biotinylated goat anti-rabbit immunoglobulin (BG) (Cedarlane Laboratories Ltd.), diluted 1:200-1:500 (optimally diluted 1:200) with 0.05 M Tris buffer, pH 7.6, rinsed with buffer, blotted, incubated for 30-60 minutes with a complex of avidin DH (or avidin D) and biotinylated horseradish peroxidase (ABC) (Cedarlane Laboratories Ltd.), each diluted 1:100-1:1000 (optimally diluted 1:100) and rinsed with buffer. Peroxidase visualization was achieved by incubating sections for 10-15 minutes in a freshly prepared, filtered solution of 0.025% DAB and 0.005% H_2O_2 in buffer. In some experiments, slides were further treated, following a distilled water rinse, by incubating them in a 1% aqueous solution of osmium for 10-15 minutes. Slides were rinsed in distilled water, dried and mounted with Permount.

FIGURE 4. Summarized ABC method of immunostaining. LM, light microscopically; EM, electron microscopically.


ABC
Diaminobenzidine
(LM visible)
Biotin
Avidin
Kallikrein
antigen
Peroxidase
(invisible)
Rabbit
anti-kallikrein
Goat
anti-rabbit
(Biotinylated)
Osmium Tetroxide
(LM&EM visible)

Semi-thin resin-embedded tissues and antisera tested with the ABC method were processed as follows. The cat submandibular gland was tested with αCSK and guinea-pig submandibular gland with αGPSK and αCSK. The cat colon and rat colon were tested with αCSK, the cat stomach was tested with αCSK or αCGK and the rat stomach was tested with αCSK. Cat trachea was examined with αCSK and GPCG and GPPG were tested with either αCGK or αNGF. Cat stomach sections were also stained for carbohydrates by the PAS method following ABC immunostaining. Light microscopic structure was examined by staining methods outlined before.

ii. Ultrathin resin sections

Ultrathin sections were cut, xylene-expanded, mounted on 150 or 200 mesh, Formvar-coated, nickel grids and processed by the ABC method. In some cases, digestion with 0.5% trypsin was performed in 0.05 M Tris buffer, pH 7.6, with 0.1% $CaCl_2$ for 10-20 minutes at 37°C. Grids were rinsed in buffer, distilled water and incubated at room temperature with normal goat serum for 5 minutes. They were blotted and submerged in drops of immune or normal rabbit sera (diluted 1:1000-1:5000) in a humidity chamber. GPCG and GPPG were also incubated in antisera containing 0.1% saponin, used to aid antibody penetration and immunostaining. Incubations at 4°C were performed for 48-72 hours. Grids were rinsed in Tris buffer, blotted, floated for 5 minutes on normal goat serum, blotted, incubated for 5 minutes on BG (diluted 1:200), rinsed with buffer, blotted, floated on normal goat serum for 5 minutes, blotted, incubated for 5 minutes in ABC (components diluted 1:100), rinsed with buffer and loaded into a multiple grid staining unit, as before. Grids were reacted with a freshly prepared, filtered solution of 0.0125% DAB and 0.0025% H_2O_2 in buffer. The reagents were stirred while grids were incubated for 3-5 minutes. Grids were rinsed twice in distilled water, dried and floated on 4% aqueous osmium for 15-25 minutes. After being rinsed twice with distilled water, they were allowed to dry.

Using this method, cat submandibular gland tissue was tested with αCSK and GPCG and GPPG were tested with αCGK. Ultrastructure was also examined by double staining methods outlined before.

iii. Pre-embedding

GPCG or GPPG tissues were processed for pre-embedding ultrastructural immunostaining after the method of Pignal *et al.* (1982). Anaesthetized animals were perfused via the left ventricle with 50 ml of 4% PFA in 0.1 M phosphate buffer, pH 7.3, for 10 minutes. Although some animals were not perfused, all glands were removed, cut into small pieces and immersed in fixative for 2 hours at room temperature. A wash followed with three changes of buffer at 4°C. Usually the following steps were done at 4°C with solutions containing 0.1% saponin. Tissue pieces were incubated in primary antisera (αCGK or normal rabbit serum, diluted 1:100-1:5000) for 48-72 hours, washed twice in buffer, incubated in normal goat serum for 10 minutes, incubated in BG (diluted 1:200) for 1 hour, washed twice in buffer, incubated in ABC (components diluted 1:100) and washed twice in buffer. The following took place at room temperature. Peroxidase activity was developed with a freshly prepared, filtered solution of 0.025% DAB and 0.005% H_2O_2 in buffer. A buffer wash was followed by treatment of pieces with 2% osmium in 0.05 M cacodylate buffer for 1 hour. Two rinses with cacodylate buffer, for 10 minutes each, were followed by a final fixation with 3% GTA, 3% PFA in 0.05 M cacodylate buffer, pH 7.4, for 90 minutes. Pieces were washed twice with buffer, dehydrated through an ascending ethanol concentration gradient and embedded in Araldite resin via propylene oxide, as before. Ultrathin sections were cut, expanded with xylene and mounted on Formvar-coated grids before examination by electron microscopy.

c. Protein A-colloidal gold

i. Ultrathin resin sections

The method of Roth *et al.* (1978) was followed for preparation of protein A-colloidal gold complex (PAG). 1 mg of protein A (Pharmacia Fine Chemicals) dissolved in 0.2 ml of distilled water was placed in a siliconized flask to which 10 ml of colloidal gold solution, pH 6.9 (ICN Pharmaceuticals Inc.), was added. The solution was mixed and then allowed to stand for 2 minutes before adding 0.2 ml of 1% polyethylene glycol (PEG) (Sigma Chemical Co.). Centrifugation at 60,000 g took place for 1 hour at 4°C. The pellet was resuspended in 10 ml PBS, pH 7.4, containing 0.02% PEG. In some cases, sections were treated with 5% or 10% H_2O_2 for 5-10 minutes before the staining sequence began. Grids with mounted sections were incubated in 0.1 M PBS, pH 7.4, with 0.5% bovine serum albumin (BSA) (Miles Laboratories Inc.) for 5 minutes and blotted. Incubation in drops of immune or normal rabbit sera (diluted 1:100-1:10,000 in 0.1 M PBS, pH 7.4, with 0.5% BSA) for 1-2 hours at 4°C, or 12-72 hours at room temperature was followed by a buffer wash and blotting. Grids were incubated with PAG for 30-60 minutes at room temperature, washed in buffer with several changes of distilled water and dried. Before examining, grids were counterstained with 5% aqueous uranyl acetate for 60 minutes.

The PAG method was used unsuccessfully to examine cat submandibular gland with αCSK and guinea-pig submandibular gland with αGPSK. Particles of gold were distributed randomly on Formvar and resin sections. As before, ultrastructure was examined by double staining methods for the electron microscope.

3. Specificity and controls

Antibody specificities were tested by double diffusion analysis (Ouchterlony, 1958). Immunodiffusion plates were prepared with 1% agarose (Bio Rad Laboratories) in saline and wells were cut by a template kit (Miles Laboratories Inc.) so that each well held a volume of

35 μl (see Figure 2). Antisera were tested against both purified antigen and tissue extracts. Antibody specificity during immunostaining was examined in some cases by absorbing specific rabbit antisera with pure antigen or by tissue extracts before use (Schachter *et al.*, 1983a). High dilutions of antisera and replacement of immune sera with normal rabbit sera were used to illustrate antibody specificity. As controls for method specificity in the PAP method, either FAB, PAP or H_2O_2 were omitted from the staining sequence.

E. Microscopy and Photography

A photomicroscope II (Carl Zeiss) was used for light microscopy and tissues were photographed with either Panatomic X black and white or Ektachrome 64 color films (Kodak Canada Inc.). Tissues for electron microscopy were viewed with a Philips 300 electron microscope at 60 or 80 kv and photographed with Kodak fine grain positive 35 mm film. Printing of black and white negatives was on Ilfospeed 4 or 5 medium weight paper and color film was printed using Cibachrome paper and chemicals.

III. RESULTS

A. Summarized Results of Immunostaining

Table 1 summarizes results of immunostaining various tissues which were fixed in several ways. Only wide classes of fixation are mentioned. Details of fixation procedures or exact concentrations of reagents are not included.

Two types of fixation were generally more successful than others in preserving antigenicity and structure. These were freeze-drying and CDI. CDI also appeared to have better success in preserving ultrastructure than freeze-drying. The next most successful type of fixation was 0.5% osmium. Higher concentrations of osmium were much less successful since antigenicity was lost. The last group of fixations, PFA-GTA combinations, were moderately successful, especially in the cat submandibular gland. It is interesting that in some tissues, such as GPCG and GPPG, only freeze-drying or CDI fixation was successful. Even 0.5% osmium, which worked for other tissues on which it was tried, did not work for the male sex glands. In conclusion, some fixations were good for immunostaining all tissues but others were suitable for only some tissues.

B. Localization of Kallikrein

1. Submandibular Gland - Light and Electron Microscopy

a. Cat

In paraffin-embedded tissue, kallikrein was localized with αCSK by the PAP method (Figures 5 and 6) Specific staining was seen only in apical regions of striated duct cells, which was similar to the findings of Schachter *et al.* (1980). This tissue was also embedded in Araldite resin and immunostained by the PAP method (Figures 7 and 8). This semi-thin section method was an improvement over the paraffin-embedded system for resolving both histological

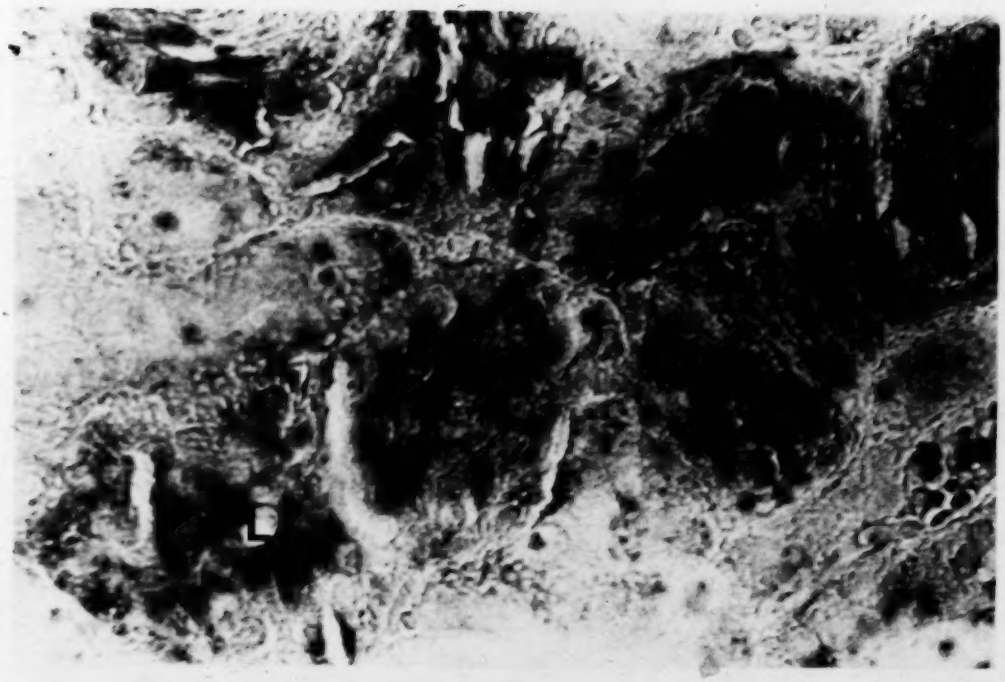
TABLE 1. SUMMARIZED RESULTS OF IMMUNOSTAINING TISSUES FIXED IN VARIOUS WAYS (BY LIGHT MICROSCOPY)

Tissue	Osmium 0.5%	Osmium 1.0%	Freeze dried	CDI	PFA-GTA
CSG	na	-	++	+++	+++
GPSG	na	na	+	++	++
CC	+	-	++++	++++	+
RC	+	-	++++	+++	++
CS	++++	+	+++	na	-
RS	+++	+	+	+++	-
CRT	++	na	na	+	+
GPCG	-	-	+++	++	++*
GPPG	-	-	++	++	-

+ (range + to ++++): Intensity of specific immunostain
-: No specific immunostain
CC: Cat colon
CRT: Cat respiratory tissue
CS: Cat stomach
CSG: Cat submandibular gland
GPCG: Guinea-pig coagulating gland
GPPG: Guinea-pig prostate gland
GPSG: Guinea-pig submandibular gland
RC: Rat colon
RS: Rat stomach
na: Experiment not attempted
*: With PVVP treatment

FIGURE 5. Kallikrein localization in apical regions of striated duct cells of the cat submandibular gland. Tissue was fixed in 1% CDI, 0.5% GTA and 1% PFA, embedded in paraffin and stained with αCSK (diluted 1:1000) by PAP method. L, lumen. (magnification X800).

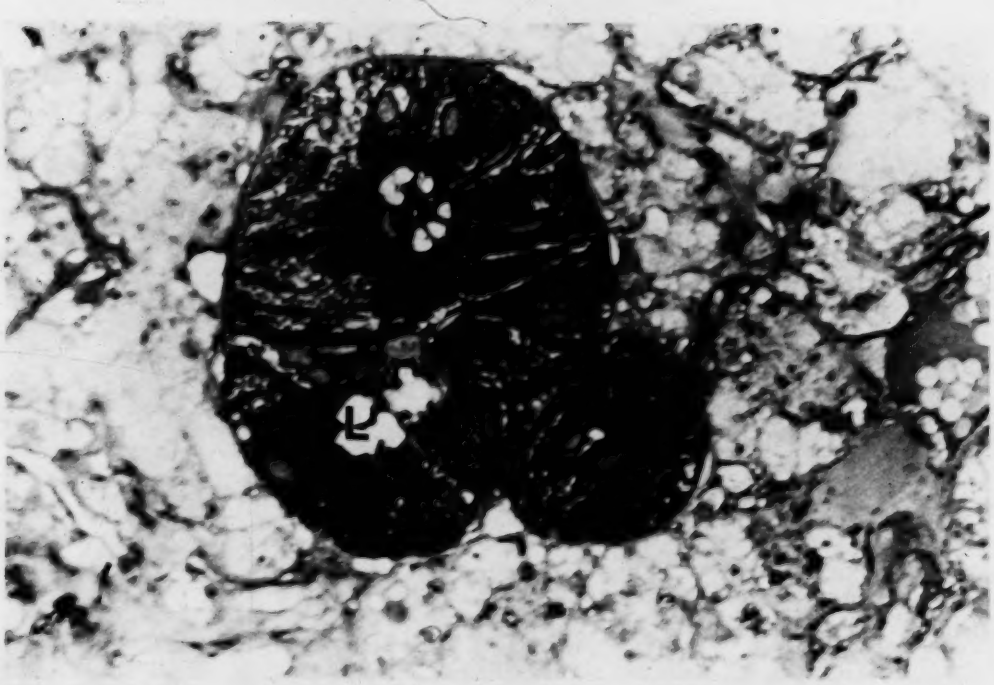
FIGURE 6. Control section for Figure 5, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X800).

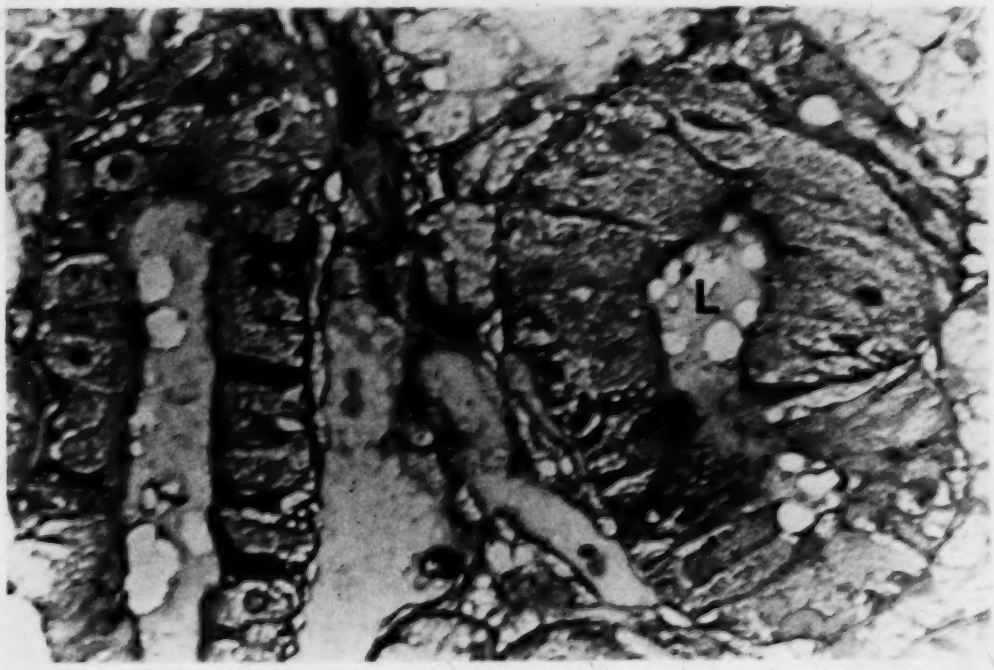

L

L

FIGURE 7. Kallikrein localization in apical regions of striated duct cells of the cat submandibular gland. Tissue was fixed in 1% CDI, 0.5% GTA and 1% PFA. Semi-thin sections of resin-embedded tissue stained with αCSK (diluted 1:5000) by PAP method. Compare resolution of immunostain to Figure 5. L, lumen. (magnification X800).

FIGURE 8. Control section for Figure 7, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X800).


L


L

structure and localization of immunostain. Specific stain was again localized exclusively in apical regions of striated duct cells. Another fixative, used to preserve adequate ultrastructure while retaining antigenicity was 1% GTA-3% PFA (Figures 9 and 10) (Schachter *et al.*, 1983c). The PAP method was used and specific localization was only in apical secretory granules of striated duct cells and not in other cells or organelles such as nuclei, mitochondria or endoplasmic reticulum. Fixation for immunocytochemistry did not preserve ultrastructure as well as routine fixation. Figure 11 shows numerous, well-preserved secretory granules and mitochondria in the apical regions of striated duct cells. In Figure 12, large numbers of apical granules were crenated and the apical cytoplasm possessed many vacuoles, perhaps due to fixation artifact. Immunostaining of this region showed granules stained so as to give a studded appearance (Figure 13) characteristic of PAP complexes (Sternberger, 1979). Some intergranule deposition was observed, indicating the presence of intergranule antigen, reaction product effusion artifact or diffusion of antigen out of disrupted granules which was subsequently stained.

Resin-embedded tissue retained antigenicity more satisfactorily in several cases than did paraffin-embedded tissue. Tissues fixed in 2% CDI, 0.5% GTA and 1% PFA and embedded in either paraffin or resin were immunostained. Surprisingly, when tissues were fixed similarly, except with a 15 minute, 1% osmium postfixation step, only resin-embedded tissue retained antigenicity with light (Figures 14 and 15) or electron microscopy (Figures 16 and 17). Paraffin-embedded tissues did not immunostain specifically. Semi-thin or ultrathin sections, fixed in this way, had similar immunostain patterns to sections fixed by other means. In light microscopy, only apical regions of striated duct cells stained. Also, ultrastructurally, only apical granules of striated duct cells stained specifically.

Diffuse and variable reaction product was seen on occasion in acinar cell granules. This happened with both immune and control sera, which supported the view that it was nonspecific. Because sodium ethoxide, used to etch sections, greatly diminished this staining, it was probably related to immunoglobulin-resin interactions. Endogenous peroxidase activity was

FIGURE 9. Electron microscopic immunolocalization of kallikrein in apical secretory granules of the cat submandibular gland, striated duct cells. Tissue was fixed in 1% GTA-3% PFA. Ultrathin resin-embedded tissue stained with αCSK (diluted 1:1000) by PAP method. L, lumen. (magnification X5000).

FIGURE 10. Control section for Figure 9, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X5000).

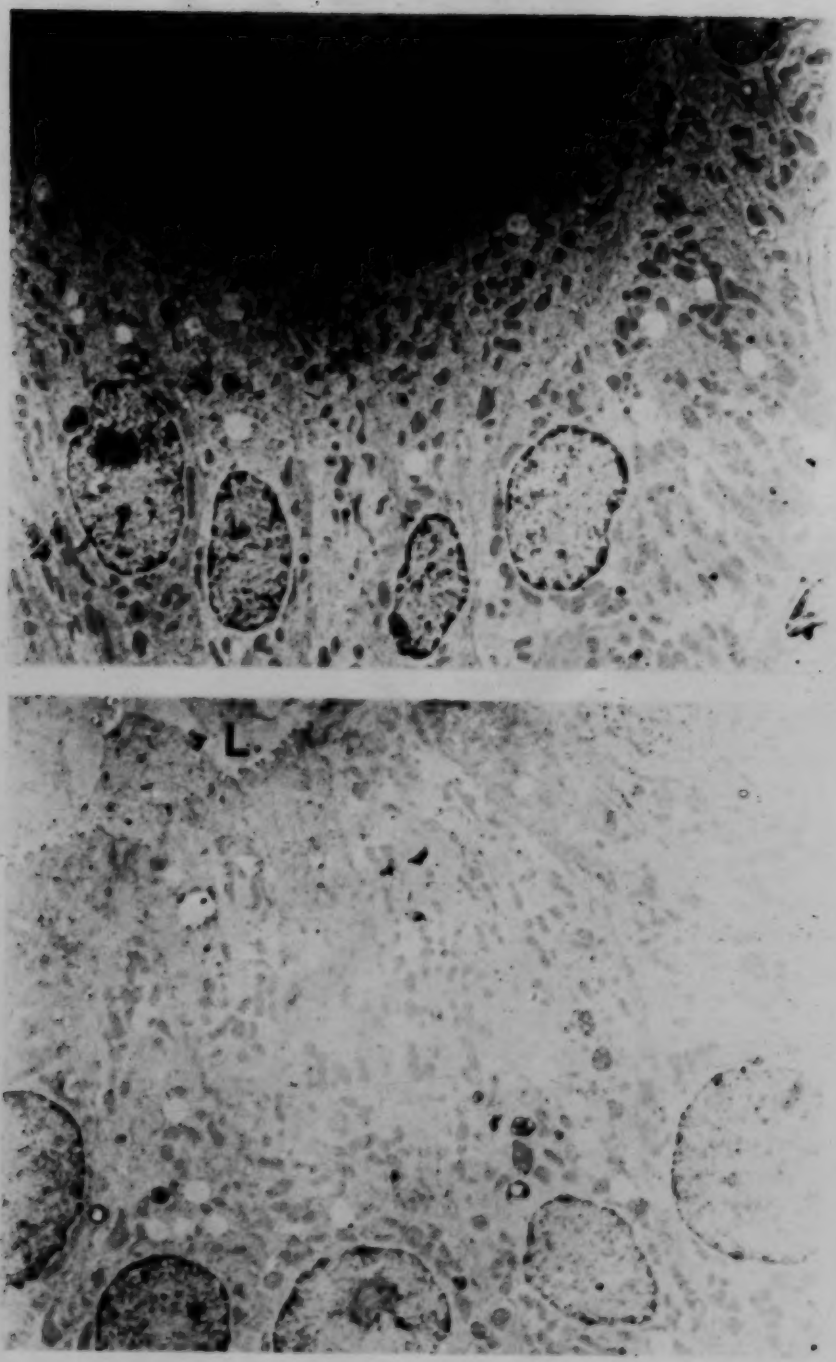

L

FIGURE 11. Ultrastructure of apical region of striated duct cell fixed by concentated aldehyde fixative (2.5% GTA, 4% PFA and 1% acrolein). Note characteristic secretion granules (arrow), mitochondria and grainy cytoplasm (magnification X40000).

FIGURE 12. Ultrastructure of apical region of striated duct cell fixed by less concentrated immunocytochemical fixative (1% GTA-3% PFA). Note crenated secretion granules (arrow) and numerous vacuoles (V) in cytoplasm compared to Figure 11 (magnification X40000).

FIGURE 13. Localization of kallikrein in apical granules stained as in Figure 9, fixed as in Figure 12. Note studded appearance of PAP reaction product over secretion granules (arrow) (magnification X40000).

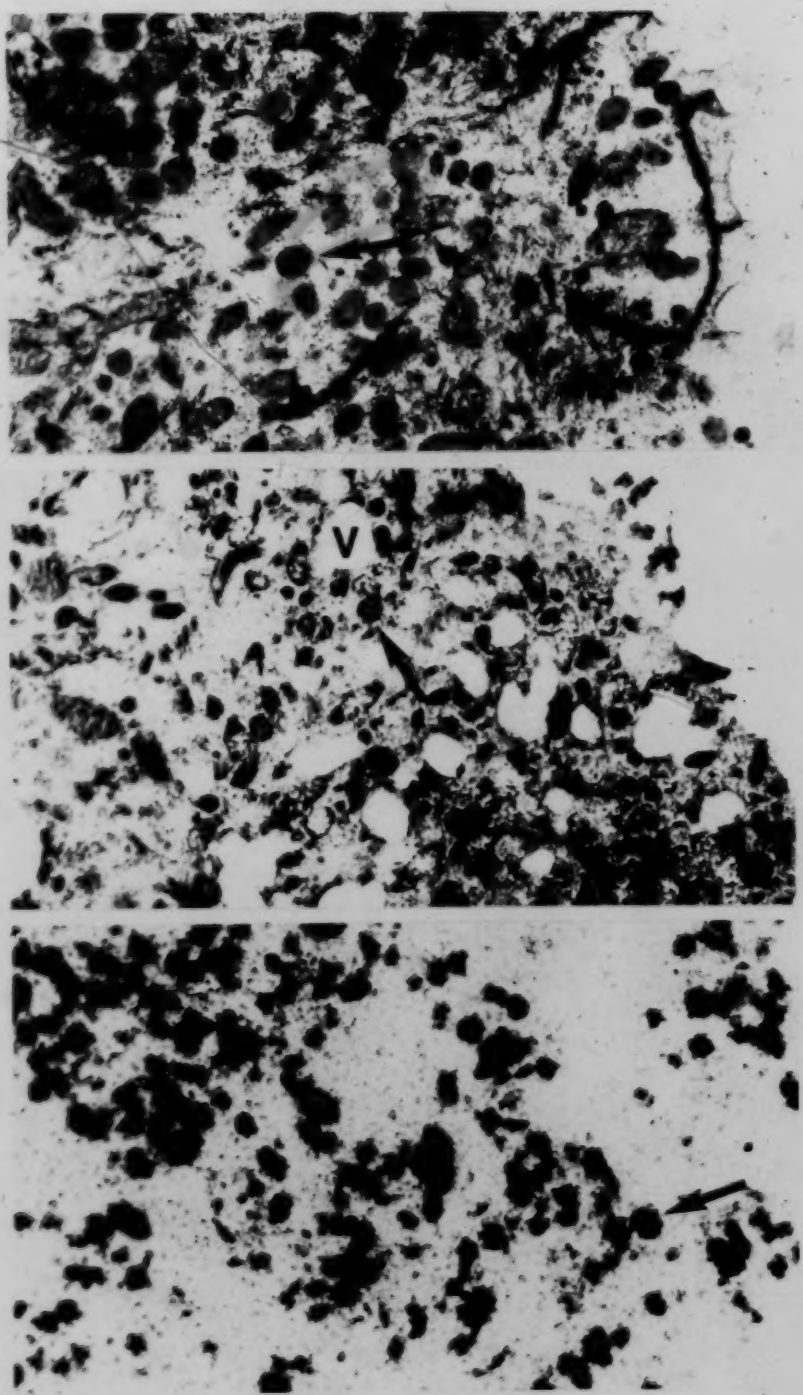
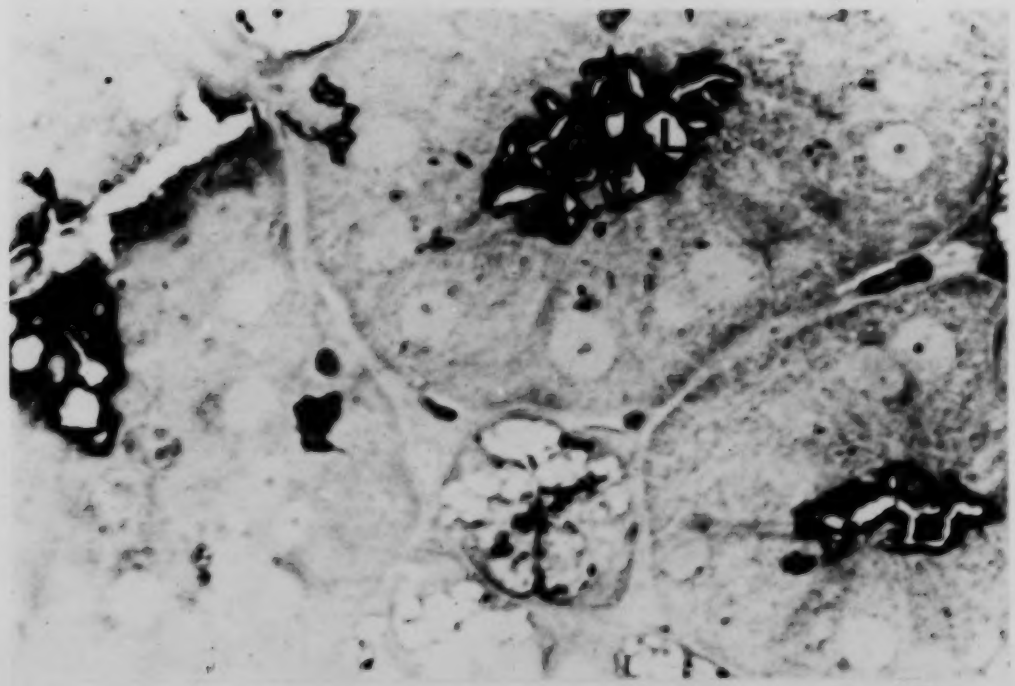

V

FIGURE 14. Kallikrein localization in apical regions and secretions of striated duct cells of the cat submandibular gland post-fixed with osmium. Tissue was fixed in 2% CDI, 0.5% GTA and 1% PFA with a 1% osmium post-fixation step. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:2000) by PAP method. L, lumen. Compare to Figure 7 (magnification X800).

FIGURE 15. Control section for Figure 14, incubated with normal rabbit serum (diluted 1:2000). L, lumen. (magnification X800).


L

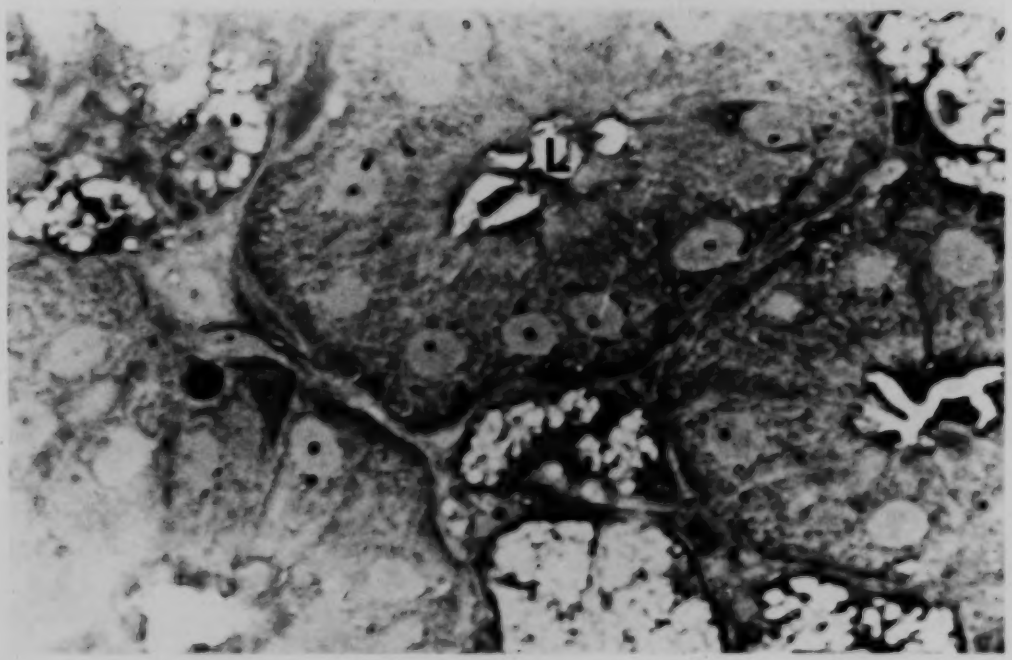
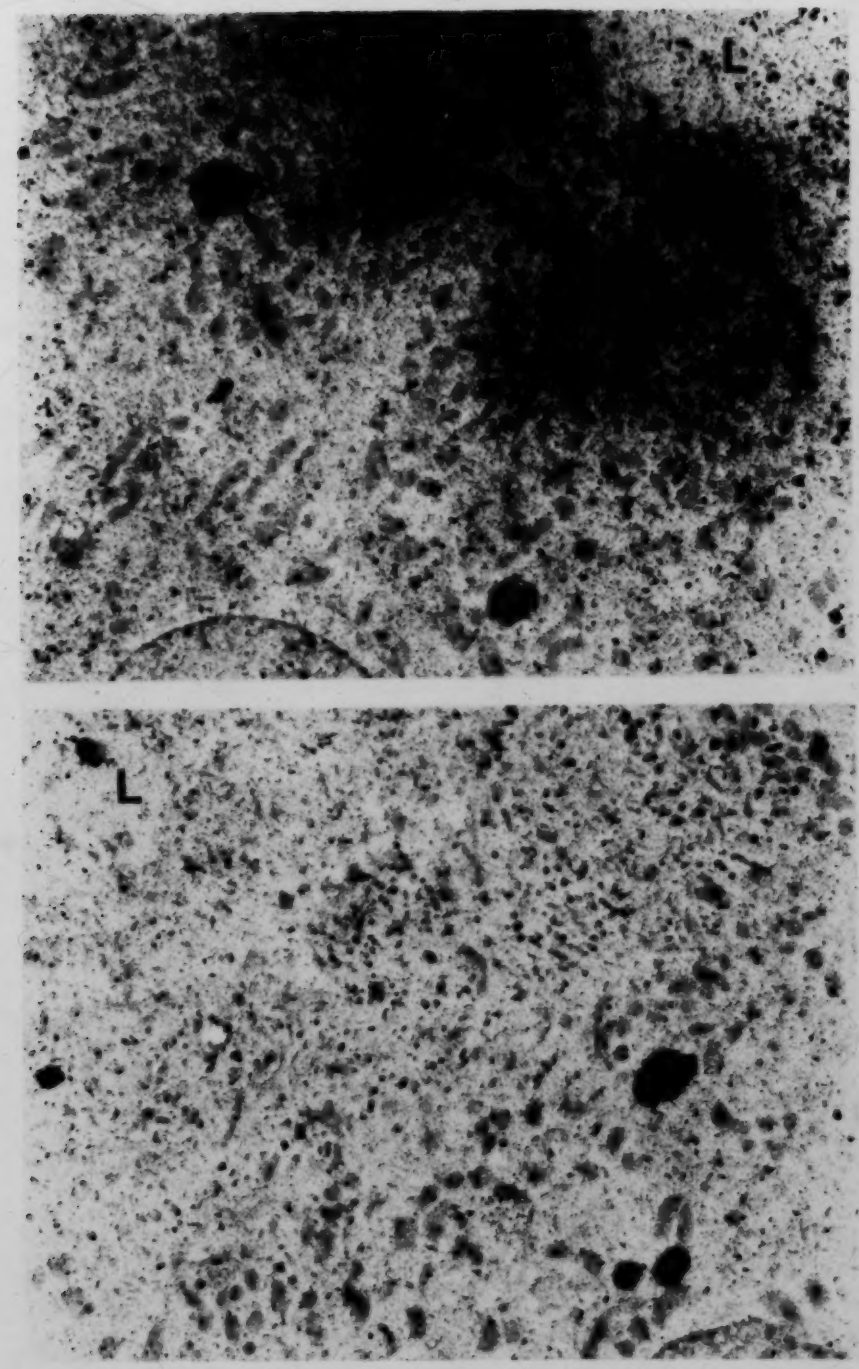

L

FIGURE 16. Electron microscopic localization of kallikrein in apical granules of cat submandibular gland, striated duct cells, fixed as in Figure 14. Ultrathin resin-embedded tissue stained with αCSK (diluted 1:2000) by PAP method. L, lumen. Compare to Figure 9 (magnification X8000).

FIGURE 17. Control section for Figure 16, incubated with normal rabbit serum (diluted 1:2000). L, lumen. (magnification X8000).


L
L

ruled out since methanol-H_2O_2 treatment did not reduce acinar staining.

b. Guinea-pig

Specific immunostaining was in apical granules of guinea-pig submandibular gland striated duct cells (Figures 18 and 19) with the ultrastructural PAP method. Apical granules were less susceptible to distortion by fixative osmolarity or concentration and were easier to preserve than those of the cat submandibular gland.

Because the semi-thin resin section method correlated with ultrastructural experiments in the cat submandibular gland and had good resolution, it was used almost exclusively for light microscopy in the guinea-pig submandibular gland. Paraffin-embedded tissue was only used to confirm αGPSK activity and the PAP system used earlier by Schachter *et al.* (1980). Different fixatives, embedding media, and immunostains developed patterns of immunostain which were similar to the cat submandibular gland. Our concentrated aldehyde-based fixative produced guinea-pig submandibular gland ultrastructure which was "normal" and similar to that of Schachter *et al.* (1983b). When concentrations of cross-linking reagents were reduced, both ultrastructure and antigenicity were retained. Etching of resin for ultrastructural studies was not always done and had no obvious effect. Endogenous peroxidase activity, known to be present in acinar granules (Bloom *et al.*, 1974), was absent in either sodium ethoxide-etched semi-thin resin sections or unetched ultrathin sections. In ultrathin PAP localizations, αGPSK stained apical granules of striated ducts better than αCSK.

2. Colon

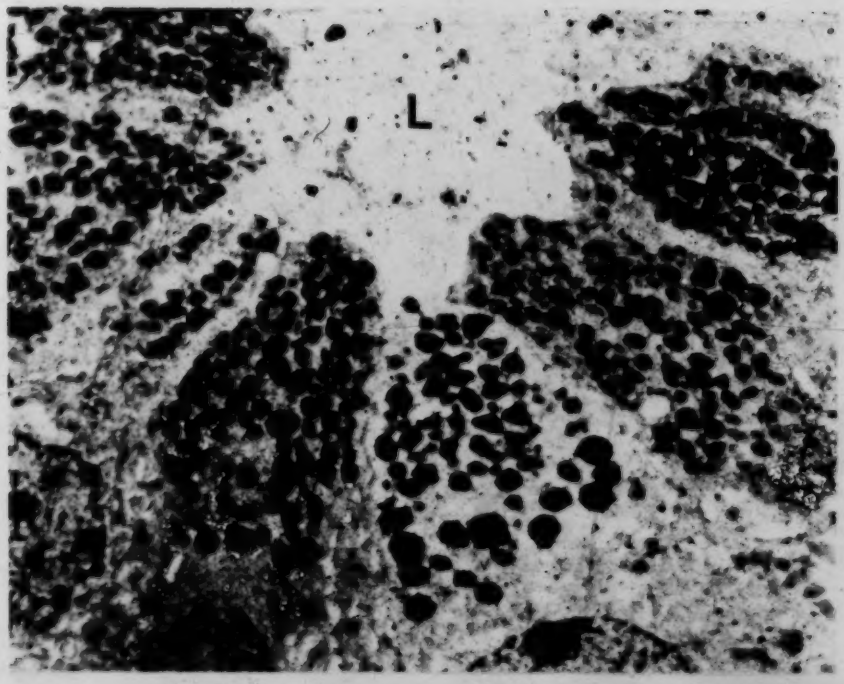
a. Cat

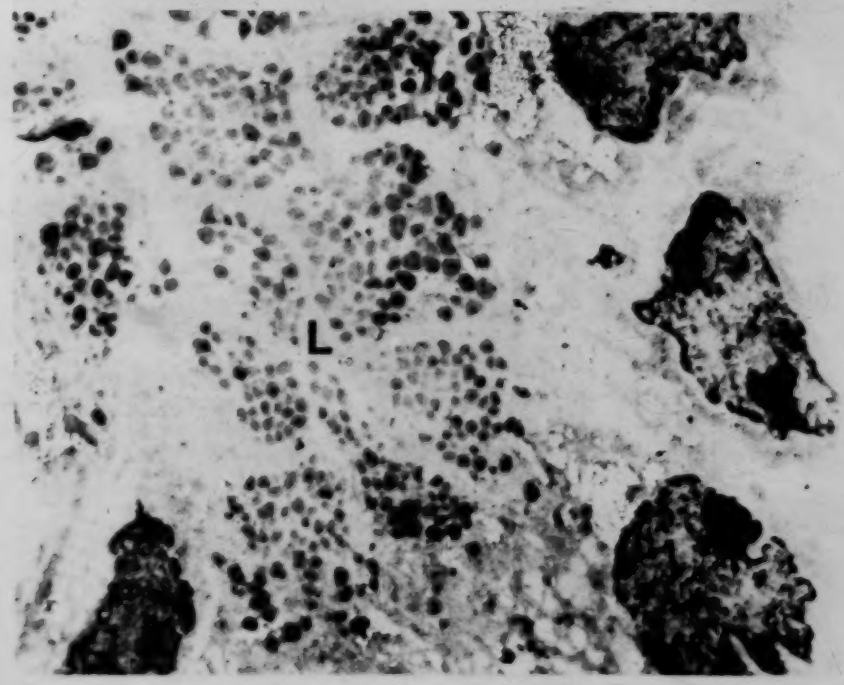
Specific staining of kallikrein was seen only in mucous (goblet) cells of the cat colon, though not all mucous cells stained. No staining was observed in columnar epithelial cells, lamina propria or non-mucosal structures. Several different immunocytochemical methods were

FIGURE 18. Electron microscopic localization of kallikrein in apical secretory granules of the guinea-pig submandibular gland striated duct cells. Tissue fixed with 5% CDI and 0.5% GTA, embedded in resin and stained with αGPSK (diluted 1:1000) by PAP method. L, lumen. (magnification X8000).

FIGURE 19. Control section for Figure 18, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X8000).

1





used.

Paraffin-embedded sections, stained by the PAP method, showed specific immunostaining in mucous cells or in their luminal discharges (Figures 20 and 21). Some mucous cells were discharging or had apparently discharged their contents at the time of fixation, and therefore did not stain. Secretion may have occurred physiologically or may have been due to an artifact of fixation or embedding. Fixation of this tissue was optimal with 1% CDI. Other fixatives for paraffin-embedded tissues, which produced specific staining, were not useful due to weak reactions or poor structure.

Semi-thin resin sections, stained by PAP or ABC methods, also showed specific staining only in mucous cells or in their luminal discharges (Figures 22 and 23). With semi-thin sections, resolution of the immunostain was better than in paraffin sections and stain was in the mucous granule compartment of goblet cells. Strands of immunostaining secretion were also observed. Furthermore, less discharge of cell contents was seen compared to paraffin-embedded tissue. Since both of these tissues were fixed with 1% CDI, the increased secretion in paraffin-embedded tissues was likely an artifact due to this embedding method. Other chemical fixatives did not produce as much specific staining as 1% CDI. PLP, PFA and osmium improved structure but reduced antigenicity. H_2O_2 etching was necessary for osmium-fixed tissue. Ethanol-fixed tissue did not retain antigenicity and saponin treatment did not increase antigenicity.

Freeze-dried tissues showed good localization of kallikrein in semi-thin resin sections (Figures 24 and 25). Specific staining was seen only in mucous cells, whether at the base or neck of crypts or at the surface. Tissues from different regions of the colon showed no difference in staining of mucous cells. Antisera and immunostaining reagents were used at optimal concentrations described before, except that primary sera in the ABC system were usually more dilute.

SDS treatment did not improve antisera or reagent penetration. All systems were strongly stained with αCSK but in the semi-thin PAP system, αRPK and αRIK also produced

FIGURE 20. Kallikrein localization in mucous cells (arrows) and secretions of cat colon. Tissue fixed in 1% CDI, embedded in paraffin and stained with αCSK (diluted 1:5000) by PAP method. L, lumen. (magnification X300).

FIGURE 21. Control section for Figure 20, incubated with normal rabbit serum (diluted 1:5000) (magnification X300).

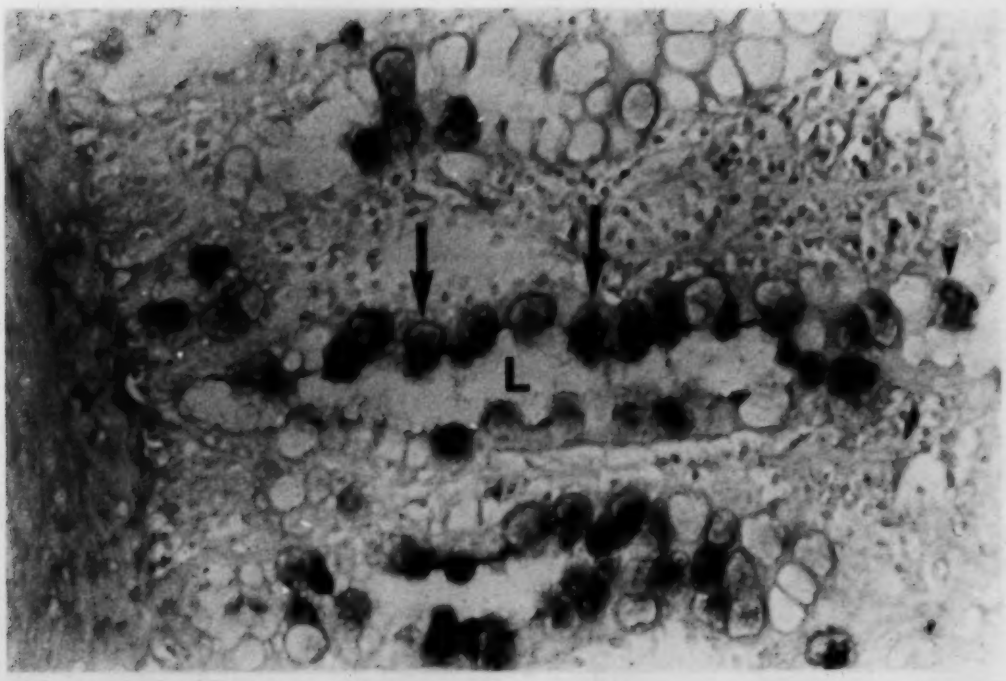
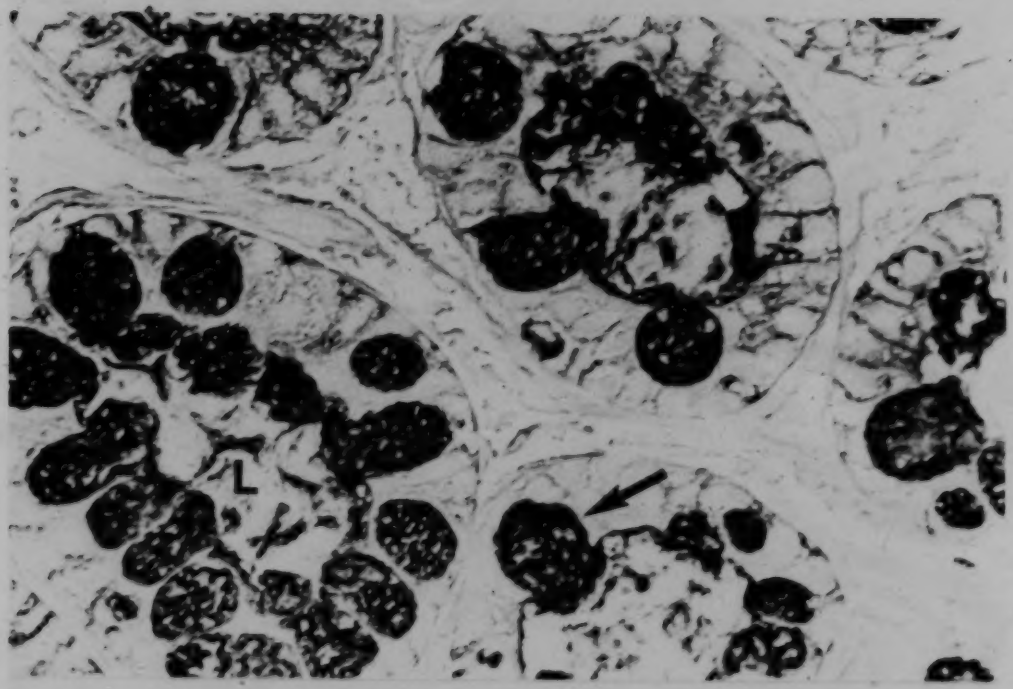

L

FIGURE 22. Kallikrein localization in mucous cells (arrows) and secretions of the cat colon. Tissue fixed with 1% CDI, embedded in resin. Semi-thin sections stained with αCSK (diluted 1:1000) by PAP method. L, lumen. Compare resolution of immunostain to Figure 20 (magnification X800).

FIGURE 23. Control section for Figure 22, incubated with normal rabbit serum (diluted 1:1000) (magnification X800).


L

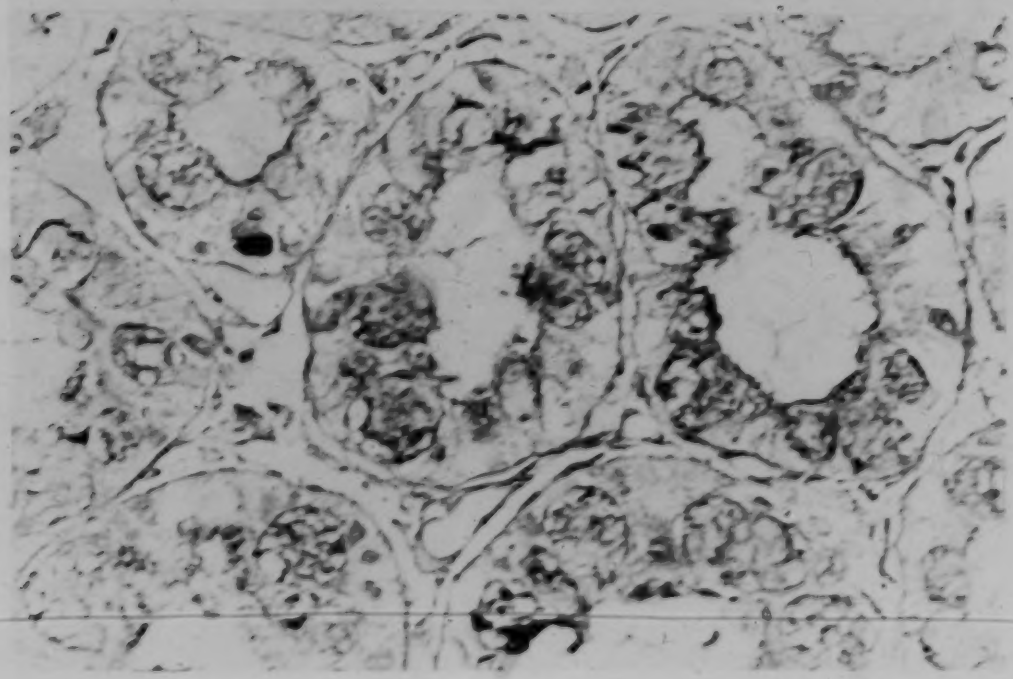
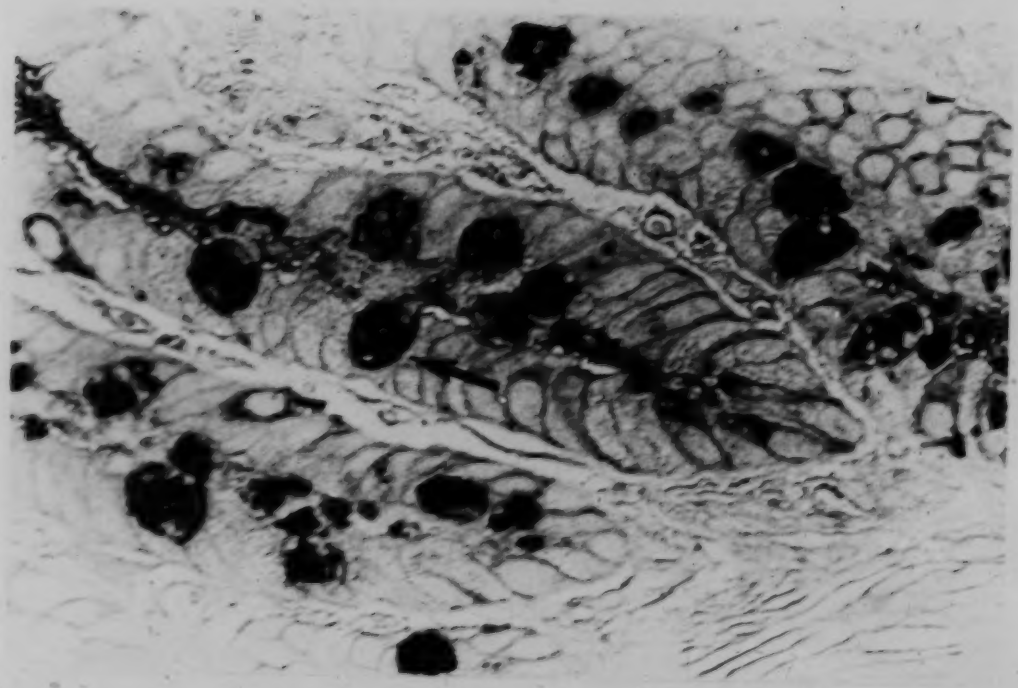


FIGURE 24. Kallikrein localization in mucous cells (arrows) and secretions of the cat colon. Tissue was freeze-dried, without fixation. Semi-thin resin-embedded sections stained with αCSK (diluted 1:5000) by PAP method. Compare to Figure 22 (magnification X800).

FIGURE 25. Control section for Figure 24, incubated with normal rabbit serum (diluted 1:5000) (magnification X800).



weak staining in mucous cells. Other antisera which produced no staining in this system were αGPSK, αHSK and αCGK. Staining was absent from sections incubated with normal rabbit sera or antisera which were preabsorbed with pure antigen or extract (Schachter *et al.*, 1980).

Ultrastructural immunolocalization of kallikrein was possible in preliminary experiments. The PAP method produced specific staining in goblet cells and in strands of secretions (Figures 26 and 27). Although ultrastructure was poor and mucous granules were completely disrupted, staining patterns supported the idea that kallikrein was associated with mucous granules. Further ultrastructural immunolocalization in the colon may require development of better fixatives than 1% CDI.

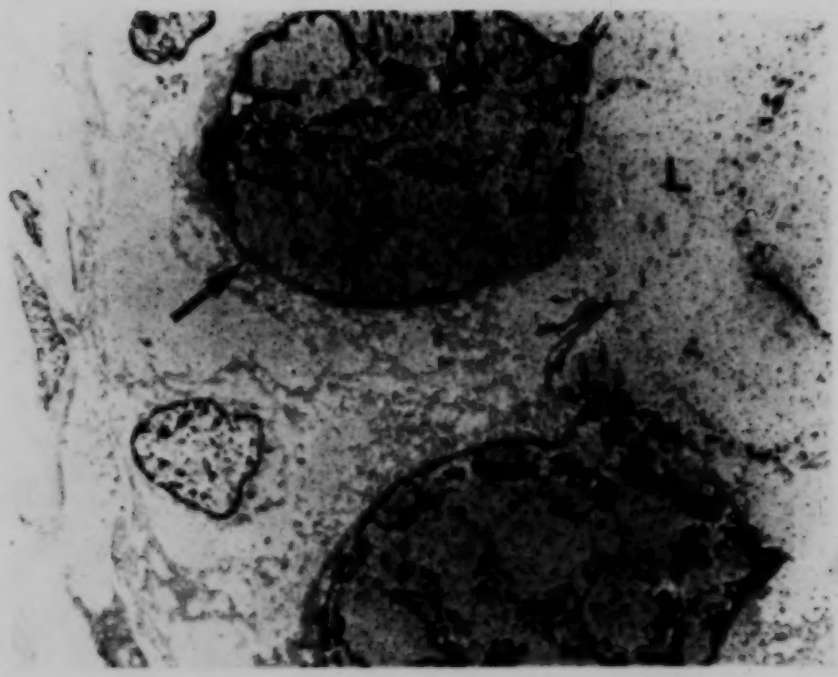
b. Rat

Kallikrein in the rat colon was localized only in mucous cells, in luminal secretions and on the surface (Figures 28 and 29). Mucous cells of ascending and descending regions of rat colon stained equally to the cat colon. Background staining in controls and immune sections was higher and reaction product often appeared in nuclear regions and in columnar epithelial cells. Mucous granule compartments of goblet cells in control sections were unstained. The ABC method produced less background stain than the PAP method, though specific staining was also reduced. αCSK primary antisera were used exclusively and at higher dilutions in the ABC method than the PAP method.

Several fixation methods preserved kallikrein antigenicity in rat colon tissue and both ABC and PAP semi-thin section methods successfully localized kallikrein. Immunostaining indicated an identical distribution of kallikrein in both cat colon and rat colon. Light microscopic structure and antigenicity were well preserved by the copper block method of freezing. Surfaces of colon often contacted the copper surface first (Figures 28 and 29) and therefore froze rapidly. Ice crystal artifact was only in areas which were not at the colon surface. Cellular detail in resin-embedded, freeze-dried, unfixed preparations was comparable to chemical-fixed tissues. Other fixatives such as 1% CDI, PLP or 2% osmium showed good structure but only

FIGURE 26. Electron microscopic localization of kallikrein in the mucous granule region of cat colon mucous, goblet cells (arrow). Ultrathin resin-embedded tissue, fixed with 1% CDI and stained with αCSK (diluted 1:5000) by PAP method. L, lumen. (magnification X5000).

FIGURE 27. Control section for Figure 26, incubated with normal rabbit serum (diluted 1:5000) (magnification X5000).



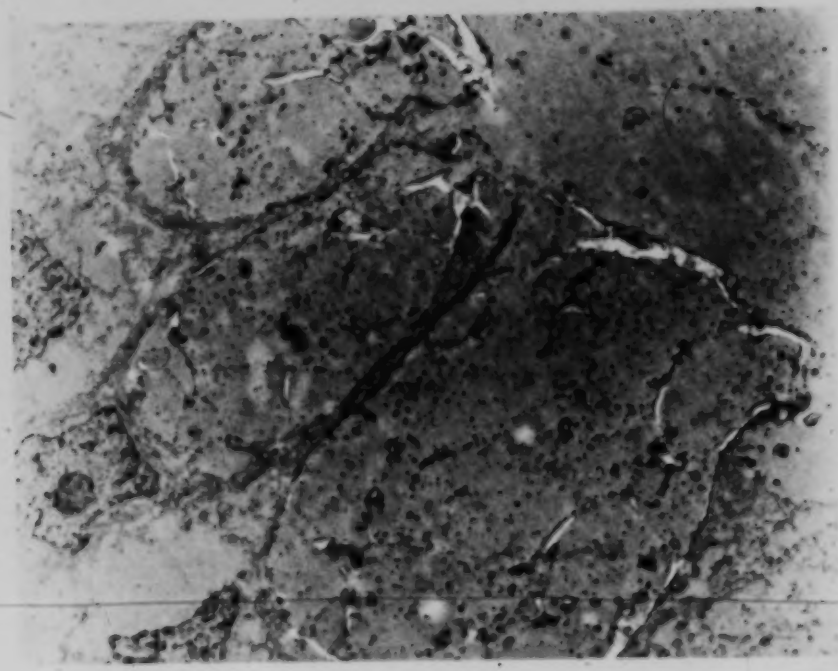
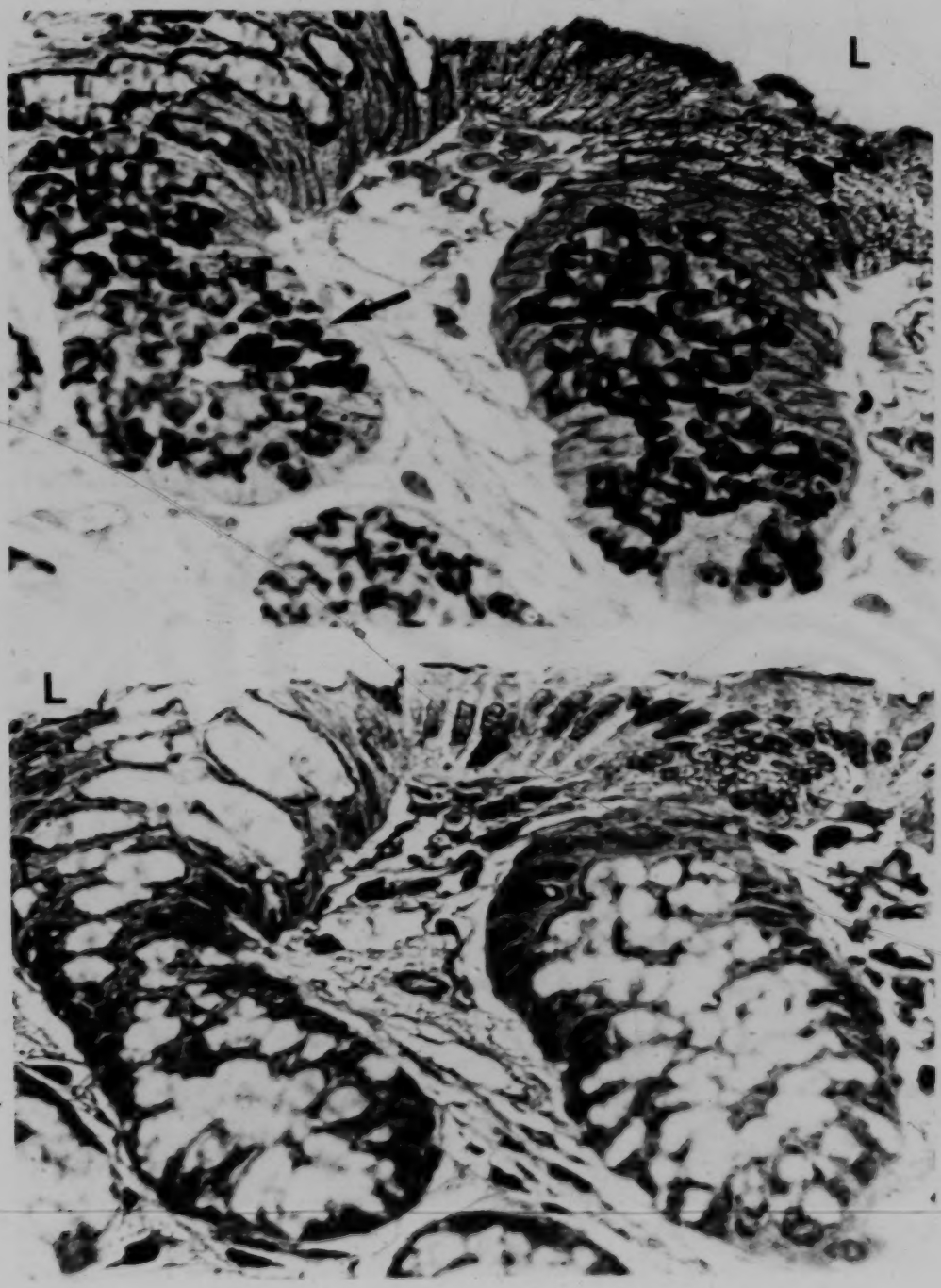


FIGURE 28. Kallikrein localization in mucous cells (arrow) of the rat colon. Semi-thin, freeze-dried, without fixation, resin-embedded tissue sections stained with αCSK (diluted 1:1000) by PAP method. L, lumen. (magnification X800).

FIGURE 29. Control section for Figure 28, incubated with normal rabbit serum (diluted 1:1000). Note high level of background stain with PAP method. L, lumen. (magnification X800).


L
L

weak antigenicity. Reducing the osmium concentration to 0.5% increased the immunostaining, making this fixative useful.

3. Stomach

a. Cat

In liquid-fixed cat stomach tissue, kallikrein was localized in surface epithelial mucous cells and in mucous cells of gastric pits, necks and glands (Figures 30 and 31). Specific staining was further resolved in apical mucuous granule compartments of these cells with semi-thin sections. Nuclear or basal regions of epithelial cells did not stain. Staining of mucous cells at the surface was usually interrupted, probably because of sloughing of apical cells.

Osmium treatment, which followed immunostaining, was used in some tissues to enhance staining which was due to peroxidase reaction product. Although osmium treatment increased background staining in control sections (Figure 31), it also enhanced the peroxidase reaction product in immune sections (Figure 30). Specific staining was relatively greater than in immune sections which were not stained with osmium (Figure 32).

Different regions of the stomach stained differently due to the pattern of mucous cell distribution. The antrum, possessing the most mucous cells, stained intensely, the corpus, with fewer mucous cells, stained less, and the fundus showed little specific staining.

The stomach antrum was optimally preserved for light microscopic structure and antigenicity with 0.5% osmium (Figures 30 to 33). Liquid fixation with other combinations of CDI, GTA or PFA were less successful due to antigen masking. Saponin addition or preliminary studies with EAI treatment did not enhance specific stain. PLP and ethanol fixatives caused loss of antigenicity. When osmium concentrations were above 0.5%, structure was improved but antigenicity was reduced.

Freeze-dried cat stomach tissue also showed immunolocalization of kallikrein in epithelial mucous cells (Figures 34 to 41). Specific staining was in the mucous granule region of

FIGURE 30. Kallikrein localization in apical regions of mucous cells (arrow) of the cat stomach antrum. Tissue was fixed in 0.5% osmium. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:1000) by ABC method with osmium post-stain treatment. L, lumen. (magnification X800).

FIGURE 31. Control section for Figure 30, incubated with normal rabbit serum (diluted 1:1000). Note background stain in mucous cells. L, lumen. (magnification X800).

L

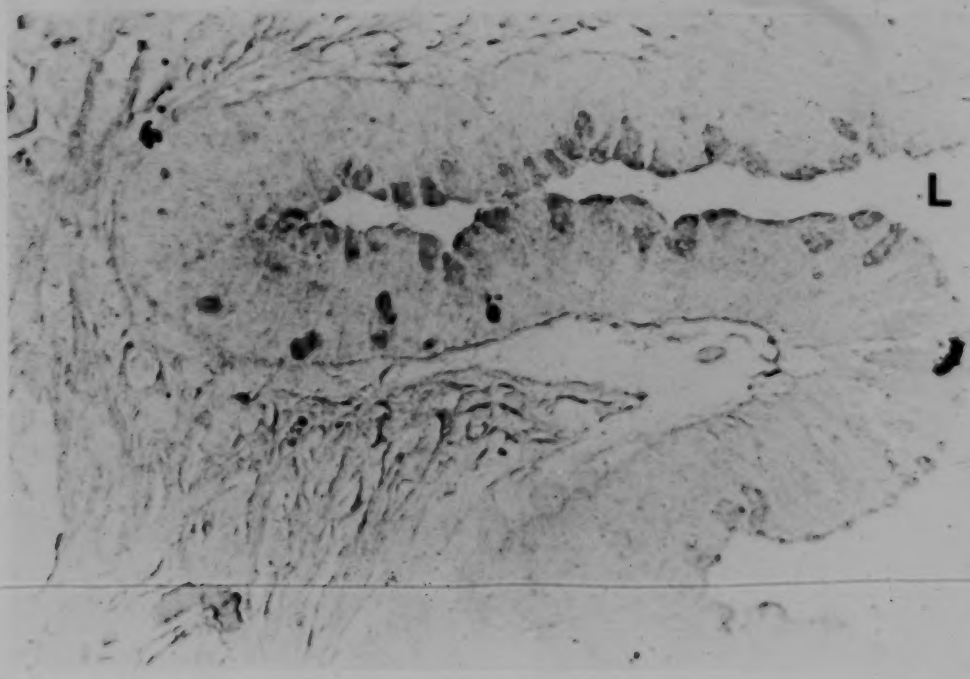

L

FIGURE 32. Kallikrein localization in apical regions of mucous cells (arrow) of the cat stomach antrum. Tissue was fixed as in Figure 30. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:1000) by ABC method without osmium post-stain treatment. L, lumen. Compare immunostain to that of Figure 30 (magnification X800).

FIGURE 33. Control section for Figure 32, incubated with normal rabbit serum (diluted 1:1000). Compare background stain to Figure 31 (magnification X800).

L

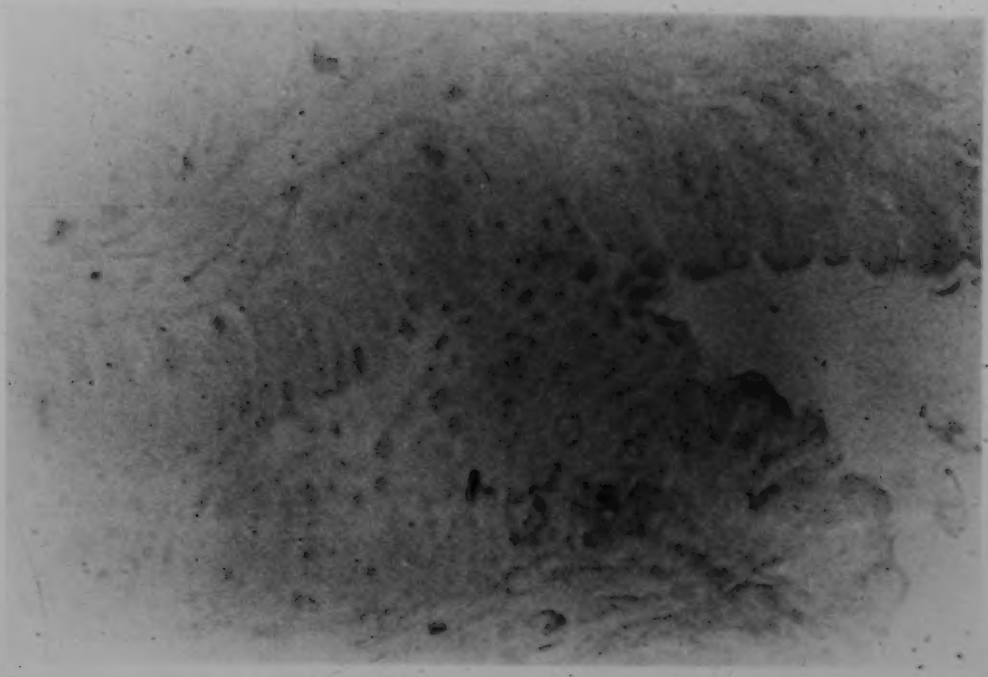


FIGURE 34. PAS stain with immunostain for kallikrein on semi-thin, freeze-dried without fixation resin-embedded cat stomach antrum. αCSK (diluted 1:1000) used in ABC method. Note kallikrein localization in periphery of mucous cells (arrow) and in secretions and PAS stain in central region of mucous cells and in secretions. L, lumen. (magnification X625).

FIGURE 35. Kallikrein localization in periphery of mucous cells (arrow) and in secretions of cat stomach antrum. Semi-thin, resin-embedded tissue stained with αCSK (diluted 1:1000) by ABC method. L, lumen. Compare immunostain to Figure 34 (magnification X625).

FIGURE 36. Control section for Figures 34, 35 and 37, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X250).

FIGURE 37. PAS stain with immunostain for kallikrein on cat stomach antrum sections. αCSK (diluted 1:1000) used in ABC method. Note that distribution of kallikrein and PAS stain were not the same. L, lumen. (magnification X250).

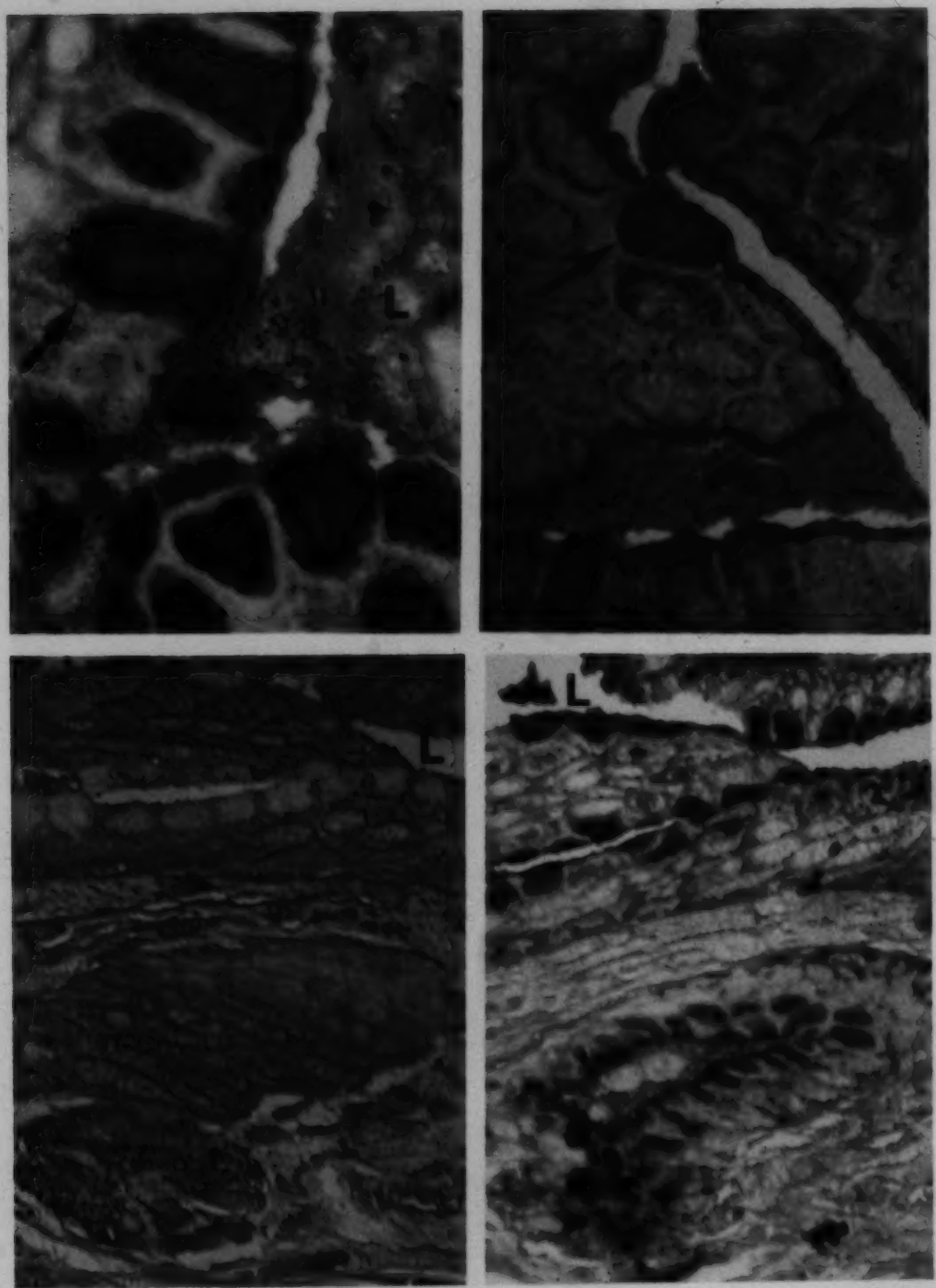
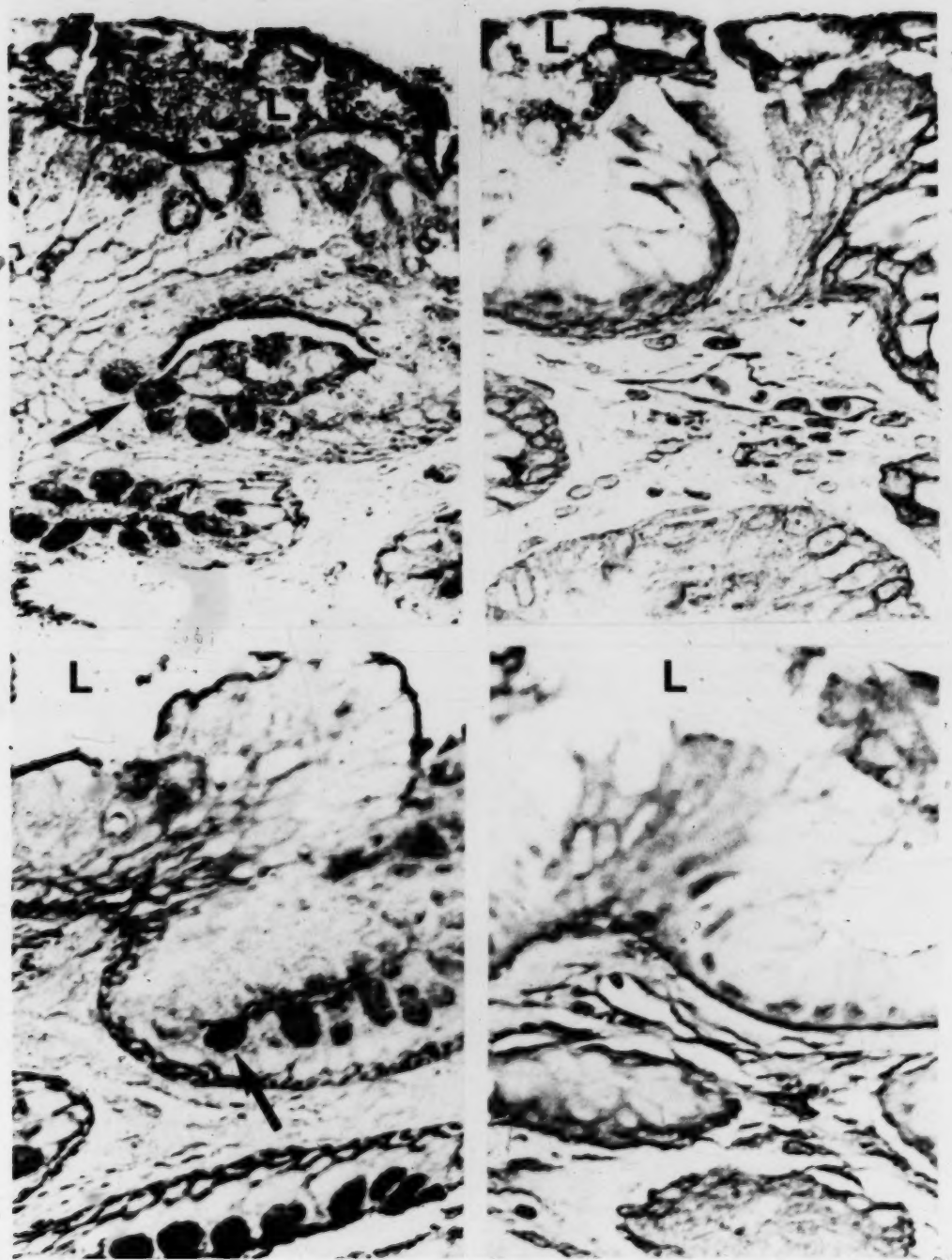

L
L
L

FIGURE 38. Kallikrein localization in the mucous cells (arrow) of cat stomach antrum. Semi-thin, freeze-dried without fixation, resin-embedded tissue stained with αCSK (diluted 1:1000) by PAP method. L, lumen. (magnification X800).

FIGURE 39. Control section for Figure 38, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X800).

FIGURE 40. Kallikrein localization in mucous cells (arrow) and surface secretions of cat stomach antrum. Semi-thin, freeze-dried, resin-embedded tissue stained with αCSK (diluted 1:1000) by ABC method. L, lumen. Compare to Figure 38 (magnification X800).

FIGURE 41. Control section for Figure 40, incubated with normal rabbit serum (diluted 1:1000). L, lumen. Compare to Figure 39 (magnification X800).


L
L
L
L

cells of the surface, pit, neck and gland, and in secretions along the surface. Copper block freezing preserved cell integrity, though tissues were deformed from their regular appearance due to the tissue's impact with the copper surface. Fixation with osmium vapor did not mask antigenicity and improved structure. Figure 35 shows kallikrein localization in surface and pit epithelial mucous cells. Staining by the PAP method was in the peripheral zone of mucous compartments of surface cells. Peripheral staining indicated that either kallikrein antigenicity was altered in the central zone of intact surface cells or secretion by surface cells caused kallikrein to be lost from central regions. Immune sections were stained with PAS to test the second possibility. PAS staining and PAP immunostaining in the same sections revealed central mucous regions of surface cells that were positive for carbohydrate but not for kallikrein (Figures 34 and 37). Some regions also had cells which reacted to either PAS stain or kallikrein immunostain, but not both (Figure 37). Kallikrein antigenicity was altered regionally within cells either as an artifact of fixation or embedding, or by intracellular changes due to chemical processing.

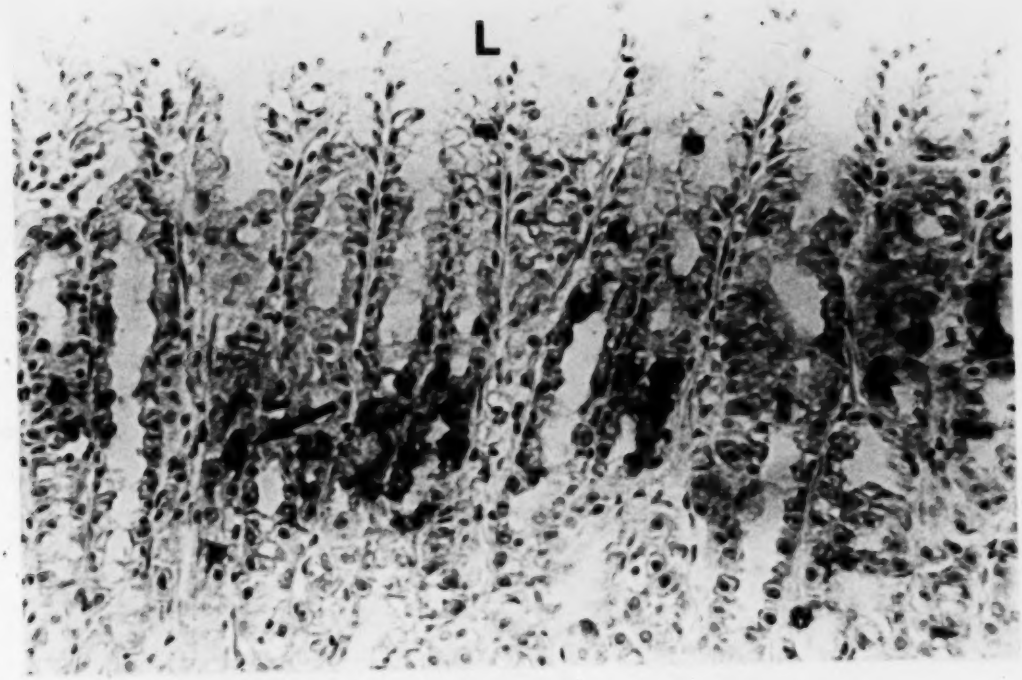
PAP and ABC immunostaining methods were compared with serial semi-thin cat stomach sections. Osmicated freeze-dried tissues, which were frozen by the copper block method, were immunostained for kallikrein by either the PAP or ABC method (Figures 38 to 41). Localization of kallikrein was in surface, pit, neck and gland mucous cells by both methods, but surface mucous staining was only in ABC-stained sections. Background staining in PAP control sections (Figure 39) was higher than in ABC control sections (Figure 41), and a better "signal to noise" ratio (i.e. specific to background staining) was obtained in the ABC method. Higher dilutions of αCSK primary antisera also produced good staining. αRPK and αCGK did not produce specific immunostaining for kallikrein in cat stomach tissue.

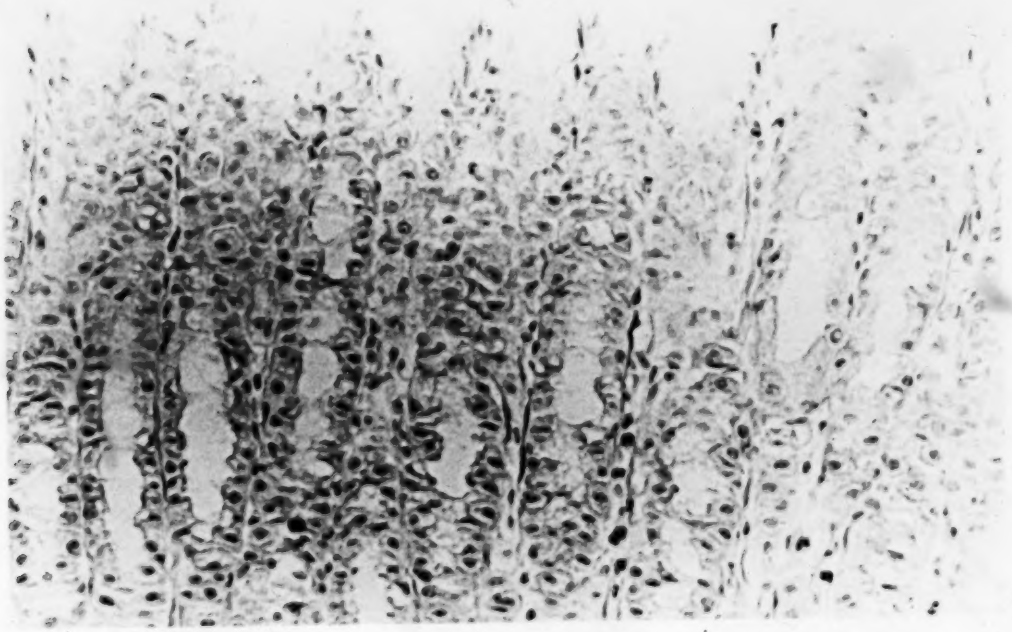
b. Rat

Paraffin-embedded tissue preserved antigenicity and specific staining, using αCSK, was found in mucous neck cells of the rat stomach (Figures 42 and 43). Staining was not found in

FIGURE 42. Kallikrein localization in mucous neck cells (arrow)of the rat stomach antrum. Tissue was fixed with 1% CDI, embedded in paraffin and stained with αCSK (diluted 1:1000) by PAP method. L, lumen. (magnification X300).

FIGURE 43. Control section for Figure 43, incubated with normal rabbit serum (diluted 1:1000) (magnification X300).


L



surface mucous cells or in mucosal secretions. αRPK and αRIK did not produce immuno-specific staining. Semi-thin resin tissue, fixed similarly, also preserved antigenicity and with better resolution. Specific staining was in the apical region of surface, pit and neck epithelial mucous cells (Figures 44 and 45). The surface mucous layer also stained specifically using either the PAP or ABC method and αCSK primary antisera. The lack of staining in surface mucous cells in paraffin-embedded tissue may have been due to loss of antigenic material during fixation or embedding.

Different immunocytochemical techniques were used to stain kallikrein in rat stomach tissue. Regional distribution of kallikrein in the rat stomach was similar to that of the cat stomach. Fixatives using higher concentrations of CDI, PFA mixtures or high concentrations of osmium did not preserve antigenicity. Semi-thin sections, fixed with 0.5% osmium, were successful in localizing kallikrein with the ABC method (Figure 46). Surface, pit and neck mucous cells stained specifically. Sections were also immunostained to compare the effects of various chemical treatments to preserve antigenicity. Enhanced staining resulted when these chemical treatments were used. EAI treatment (Figure 47) resulted in more intense staining than PVPP treatment (Figure 48). Control sections for untreated, EAI or PVPP-treated immune sections did not stain.

4. Respiratory Tract

a. Cat

Preliminary results localized kallikrein in cat trachea. Semi-thin resin sections of tissue were used which were fixed with a 1% CDI-3% PFA mixture and treated with EAI. Specific staining was localized in submucosal glands (Figures 49 and 50). Frequently, using osmium-fixed or other tissues, specific staining was in surface mucous, goblet cells. Although cat trachea had a variable staining pattern in immune sections, control sections were always devoid of stain.

FIGURE 44. Kallikrein localization in mucous cells (arrow) and surface secretions of the rat stomach antrum. Tissue was fixed in 1% CDI. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:5000) by PAP method. L, lumen. Compare resolution and distribution of immunostain to Figure 42 (magnification X800).

FIGURE 45. Control section for Figure 44, incubated with normal rabbit serum (diluted 1:5000) (magnification X800).

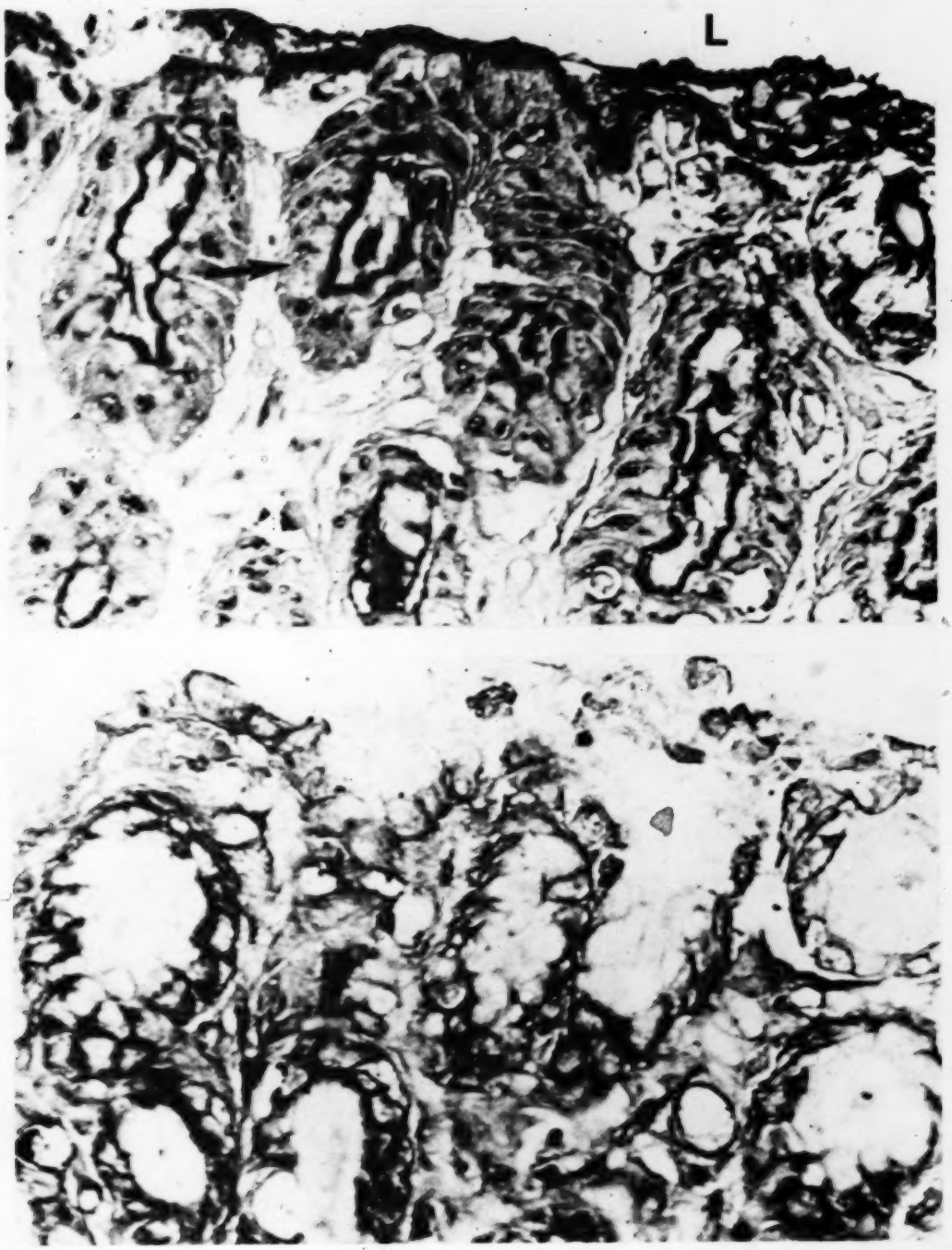

L

FIGURE 46. Kallikrein localization in apical regions of mucous cells (arrow) of the rat stomach antrum. Tissue was fixed in 0.5% osmium. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:5000) by ABC method. L, lumen. (magnification X800).

FIGURE 47. Kallikrein localization in apical regions of mucous cells (arrow) of the rat stomach antrum treated, fixed as in Figure 35, with EAI. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:5000) by ABC method. L, lumen. Compare immunostain to Figure 46 (magnification X800).

FIGURE 48. Kallikrein localization in apical regions of mucous cells (arrow) of the rat stomach antrum, fixed as in Figure 46, treated with PVPP. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:5000) by ABC method. L, lumen. Compare immunostain to Figures 46 and 47 (magnification X800).

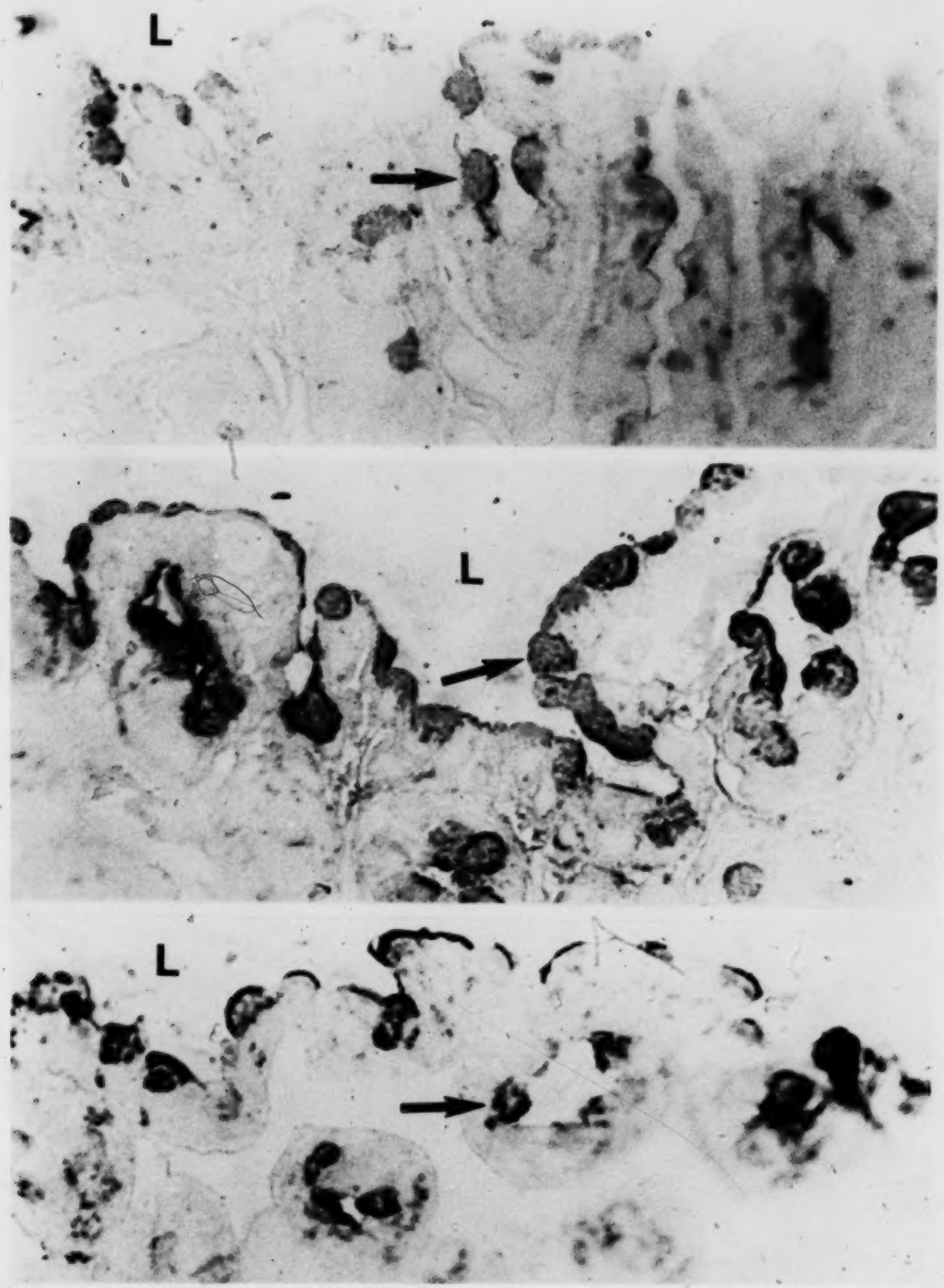
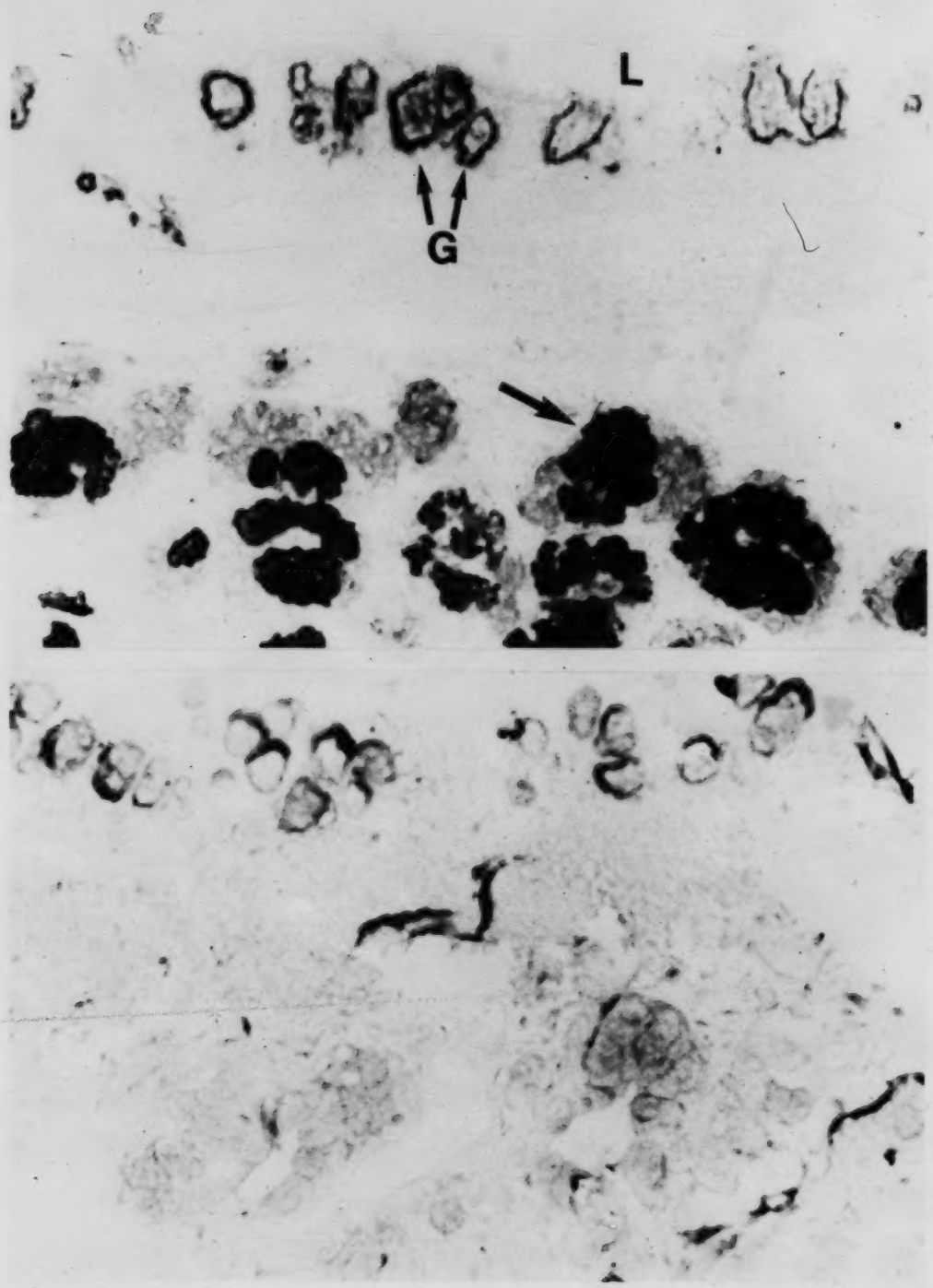

L
L
L

FIGURE 49. Kallikrein localization in submucosal glands (arrow) of cat respiratory tissue. Tissue was fixed in 1% CDI-3% PFA. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:1000) by PAP method. G, goblet cell; L, lumen. (magnification X800).

FIGURE 50. Control section for Figure 49, incubated with normal rabbit serum (diluted 1:1000) (magnification X800).


L
G

C. Localization of Kallikrein and Nerve Growth Factor

1. Male Sex Glands - Light Microscopy

a. Guinea-pig

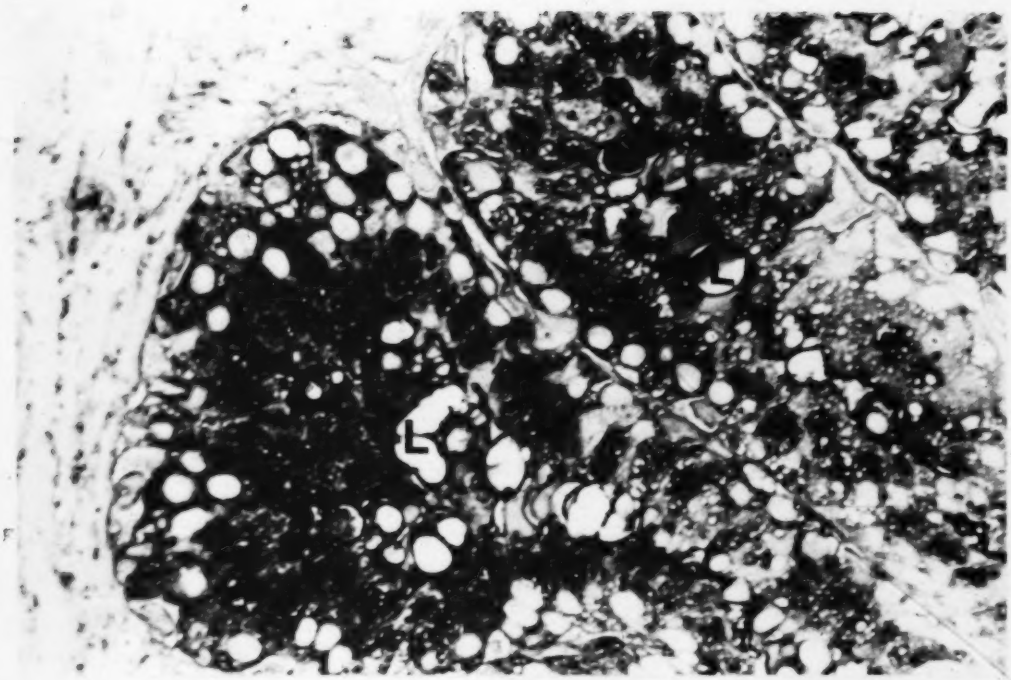
Kallikrein and NGF were localized by light microscopy in male sex glands. With αCGK antisera, kallikrein was localized in secretory epithelial cells of the GPCG and GPPG. With αNGF antisera, NGF was localized in epithelial cells of GPPG tissue. Antigen distribution depended on the fixation method.

Figures 51 and 52 show the localization of kallikrein in secretory epithelial cells. Semi-thin sections stained diffusely in apical cytoplasm and in secretions. This pattern was similar to that described by Schachter *et al.* (1978). Staining was in most cells and predominated in the granular endoplasmic reticulum-Golgi region described by Wong and Tse (1981). Peripheral, but not central staining occurred in most apical blebs, characteristic of apocrine secretory mechanisms. Specific staining was not found in nuclear, basal or non-epithelial regions. Semi-thin sections were also prepared from freon-frozen, freeze-dried GPCG tissue and immuno-stained for kallikrein (Figures 53 and 54). Distribution of specific stain was slightly different from liquid-fixed tissue. Although secretions and peripheral regions of apical blebs stained intensely, apical cytoplasmic regions of epithelial cells did not. This may have been due to large-scale ice crystal artifact present in all cells which caused a loss of antigenicity.

Antigen preservation was successful using PVPP treatment. GPCG tissue, fixed with 6% PFA and treated with PVPP, stained specifically in secretory regions of epithelial cells (Figure 55). Control sections were devoid of stain (Figure 56). Tissues that were fixed identically but without PVPP treatment did not stain. Although PVPP treatment enhanced antigenicity in tissues fixed by relatively high aldehyde concentrations (6% PFA), neither it nor EAI treatment enhanced staining in tissues which were fixed with 1% CDI-2% PFA. Fixatives containing GTA or osmium caused antigen masking though structure was improved. Other

FIGURE 51. Kallikrein localization in secretory epithelial cells of the guinea-pig coagulating gland. Semi-thin resin-embedded tissue, fixed with 1% CDI and 1% PFA and stained with αCGK (diluted 1:5000) by PAP method. L, lumen. (magnification X800).

FIGURE 52. Control section for Figure 51, incubated with normal rabbit serum (diluted 1:5000). L, lumen. (magnification X800).


L
L

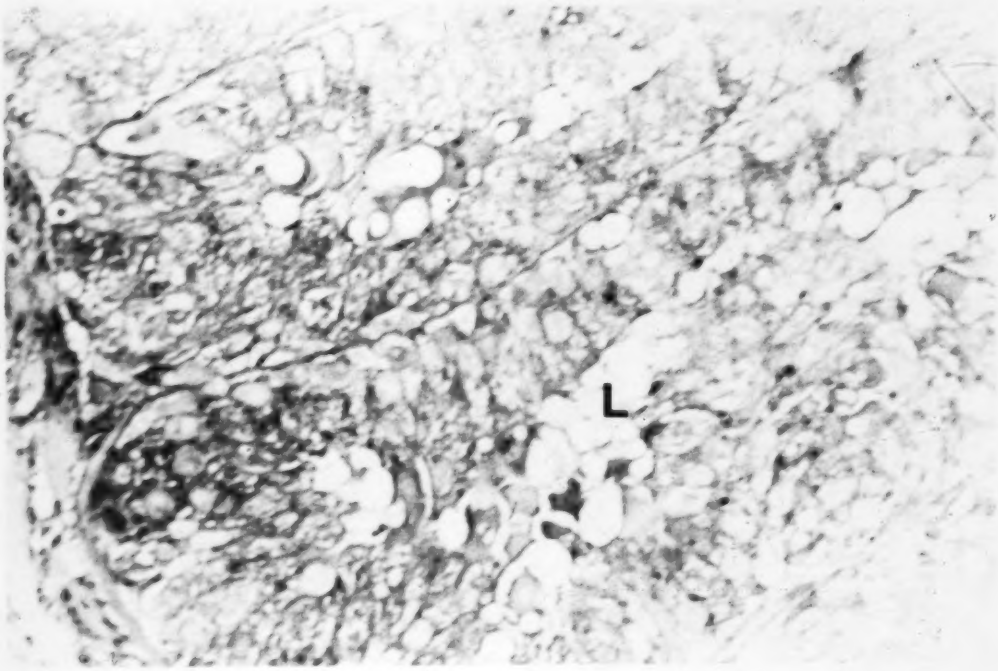
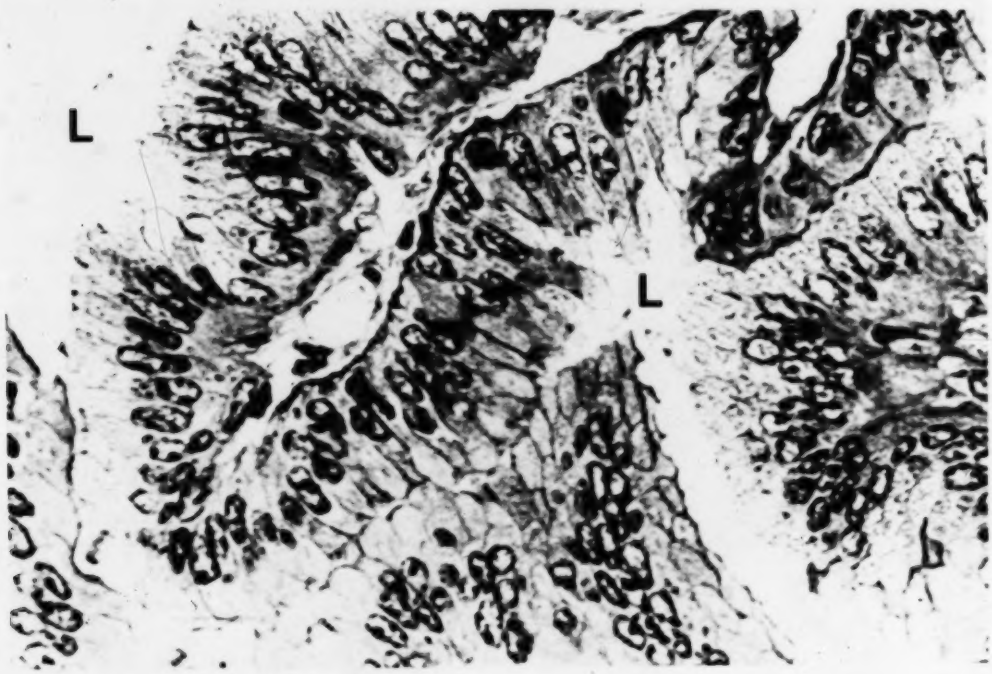

L

FIGURE 53. Kallikrein localization in secretory epithelial cells of the guinea-pig coagulating gland. Tissue was freeze-dried, without fixation. Semi-thin resin-embedded tissue stained with αCSK (diluted 1:5000) by PAP method. L, lumen. Compare to Figure 49 (magnification X800).

FIGURE 54. Control section for Figure 53, incubated with normal rabbit serum (diluted 1:5000). L, lumen. (magnification X800).

L


L
L

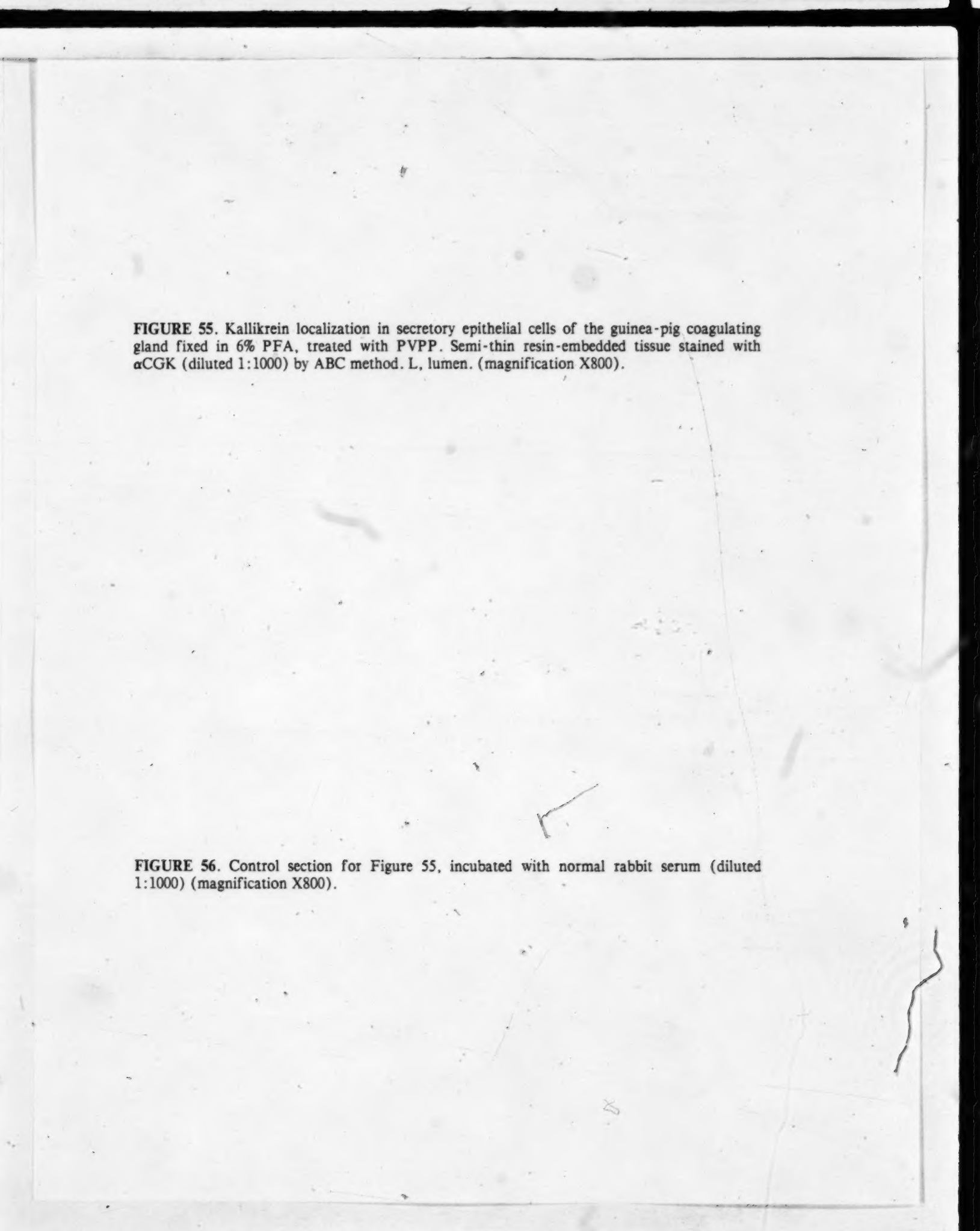
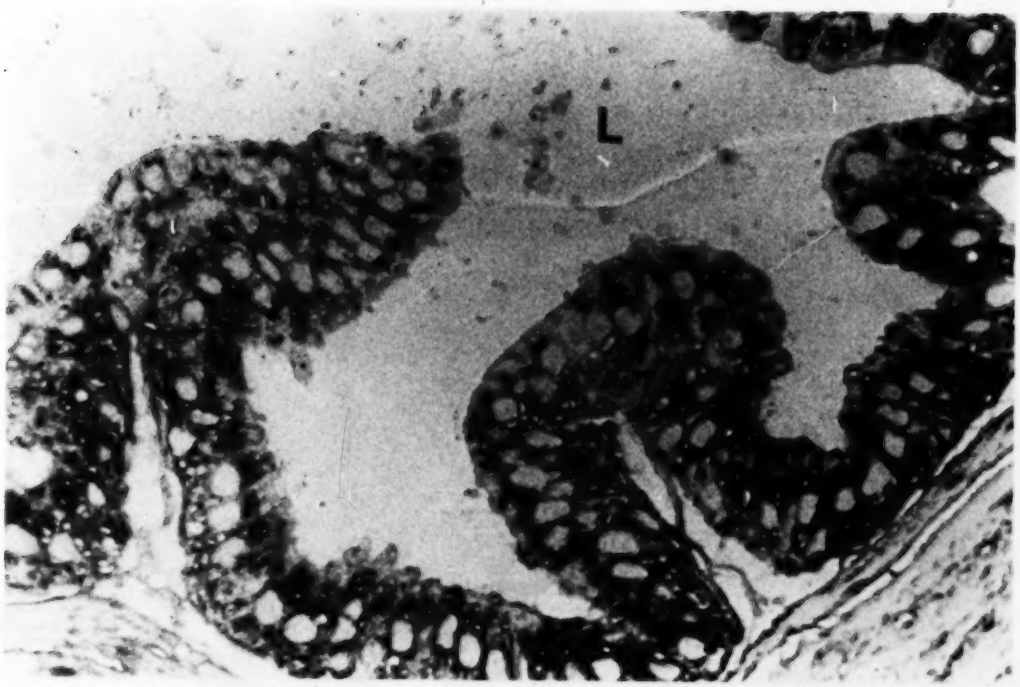


FIGURE 55. Kallikrein localization in secretory epithelial cells of the guinea-pig coagulating gland fixed in 6% PFA, treated with PVPP. Semi-thin resin-embedded tissue stained with αCGK (diluted 1:1000) by ABC method. L, lumen. (magnification X800).

FIGURE 56. Control section for Figure 55, incubated with normal rabbit serum (diluted 1:1000) (magnification X800).

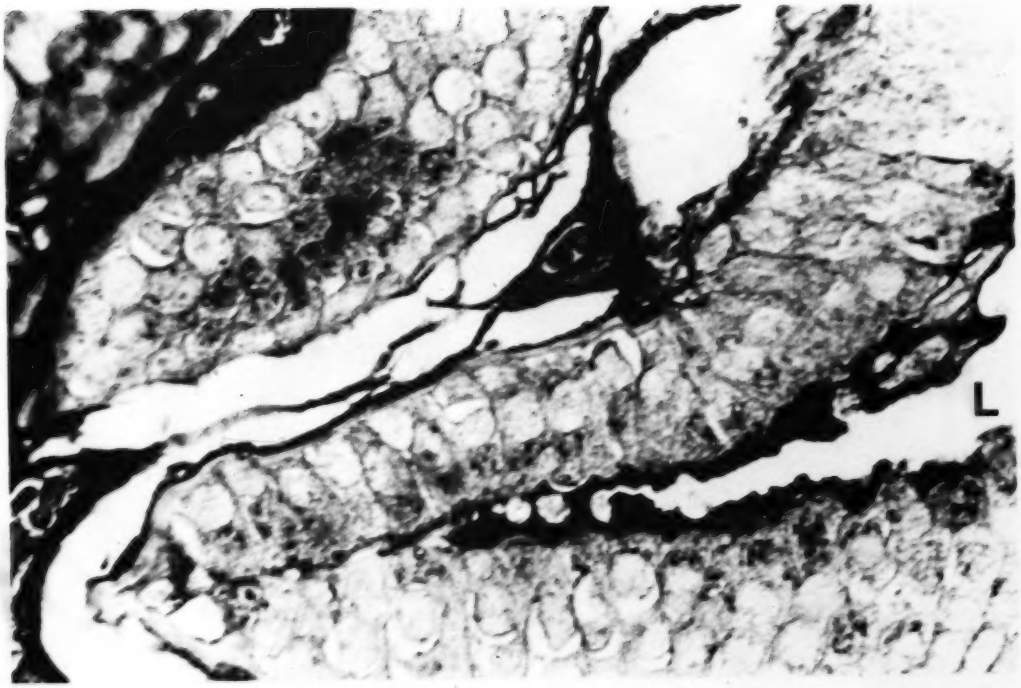

L

fixatives produced poor structure and weak antigenicity. Trypsin treatment did not unmask antigen and saponin did not enhance immunostain. αCGK produced immunostain with PAP or ABC methods, but αNGF did not immunostain the GPCG with the ABC semi-thin method. Ultrastructural immunostaining was unsuccessful despite trypsin and saponin treatments and pre-embedding immunocytochemistry. Despite semi-thin sections which immunostained, ultra-thin sections which were treated identically only had non-specific staining in immune or control sections.

Kallikrein and NGF were immunolocalized in copper block-frozen, freeze-dried GPPG tissue. Figure 57 shows specific staining in apical cytoplasm of epithelial secretory cells of the dorsal prostate. Secretions also stained. Epithelial cells of control sections were devoid of stain (Figure 58). The apical cytoplasm and secretions of epithelial cells also stained specifically with αNGF antisera (Figure 59), which was similar to the results of Shikata *et al.* (1984) using paraffin-embedded tissues. Control sections did not stain (Figure 60).

FIGURE 57. Kallikrein localization in apical cytoplasm and secretions of epithelial secretory cells of the guinea-pig prostate gland. Tissue was freeze-dried without fixation. Semi-thin resin-embedded tissue stained with αCGK (diluted 1:5000) by PAP method. L, lumen. (magnification X800).

FIGURE 58. Control section for Figure 57, incubated with normal rabbit serum (diluted 1:5000). L, lumen. (magnification X800).


L

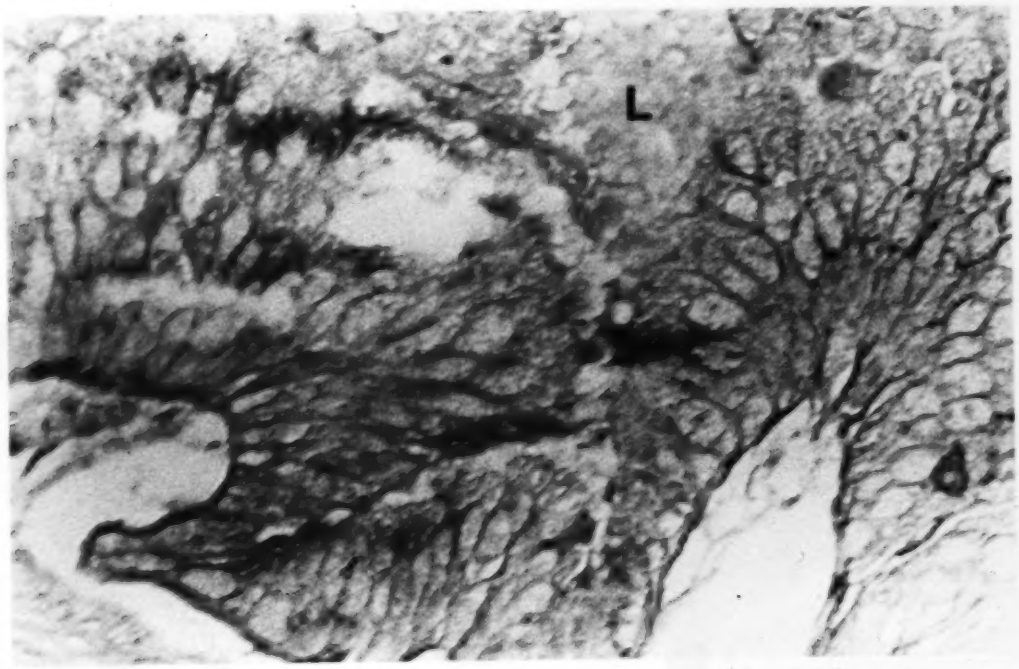
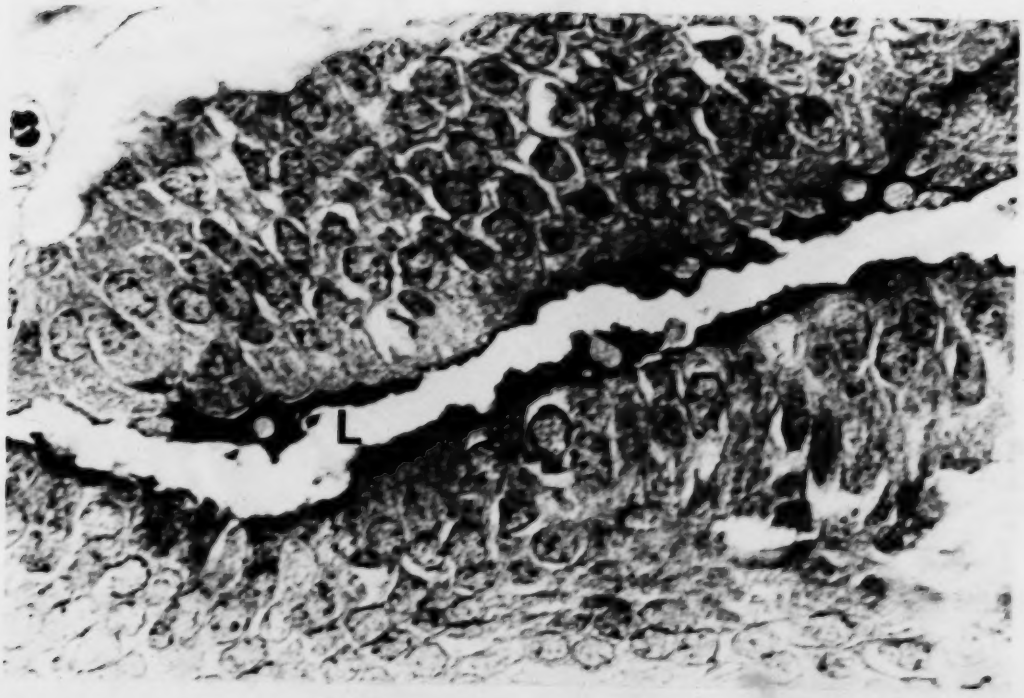

L

FIGURE 59. NGF localization in apical cytoplasm and secretions of epithelial secretory cells of the guinea-pig prostate gland. Tissue was freeze-dried without fixation. Semi-thin resin-embedded tissue stained with αNGF (diluted 1:1000) by PAP method. L, lumen. (magnification X800).

FIGURE 60. Control section for Figure 59, incubated with normal rabbit serum (diluted 1:1000). L, lumen. (magnification X800).


L

L

IV. DISCUSSION

A. Kallikrein and Related Proteases in Salivary Glands

Many chemically related proteases exist in salivary glands and saliva. Results in this thesis show the localization of kallikrein, one of these, in the apical granules of submandibular duct cells. Salivary glands from different mammals also contain various esterolytic enzymes and other macromolecules including kallikrein (Brandtzaeg *et al.*, 1976), tonin (Ørstavik *et al.*, 1982a; Zacharatos *et al.*, 1983), sialotonin, a vasopressor substance (Barton *et al.*, 1976), renin (Angeletti *et al.*, 1967; Barka, 1980; Bing *et al.*, 1980; Hiramatsu *et al.*, 1981), esteroproteases A, B, C and D (Boesman *et al.*, 1976) and other variously named serine proteases. Erythropoietin (Fava-de-Moraes *et al.*, 1979) is found in salivary gland duct cells along with AM_2, a secretory glycoprotein (Nieuw Amerongen *et al.*, 1981). Localization studies indicate that most proteases above are present in cells of the salivary gland ducts.

Many esteroproteases are found to resemble each other chemically but to possess different activities. Protein A (Bennick, 1975), a calcium-binding protein, is probably identical to protein III or IV. Protein C also resembles protein I or II. Esteroproteases A and D from mouse submandibular glands (Boesman *et al.*, 1976) are functionally separate, since protease D is thought to be identical to the epidermal growth factor (EGF)-binding protein. Tonin, identical to esterase A (Brandtzaeg *et al.*, 1976), represents 10% of the protein from rat submandibular gland (Thibault and Genest, 1981) and co-exists in the gland with kallikrein (Ørstavik and Glenner, 1978). Similarly, in mouse submandibular glands, renin and kallikrein co-exist (Barka, 1980).

Among salivary growth factors with profound effects are EGF, which stimulates the proliferation of various epithelia (Gospodarowicz, 1981), and NGF, which affects development of the sympathetic adrenergic system. EGF, isolated by Cohen (1959), was localized in secretory granules of duct cells of rodent submandibular glands (Turkington *et al.*, 1971; Gresik and Barka, 1977; Tanaka *et al.*, 1984; Murphy *et al.*, 1980).

A connection was made between salivary EGF and gastrointestinal function by the observation that this factor stimulated gastric mucosal growth (Oka *et al.*, 1983; Dembinski and Johnson, 1985). EGF from duct cells is secreted both into saliva and into the circulation (Hirata and Orth 1979; Bing *et al.*, 1980; Nexø *et al.*, 1984). Since aggressive behaviour and α-adrenergic stimulation caused stimulation of salivary secretion of EGF and renin, possibly these factors may be involved in stress and related events, e.g., wound healing.

NGF co-exists with kallikrein in ducts of submandibular glands and is induced by testosterone and thyroxine (Hendry and Iverson, 1973; Ishii and Shooter, 1975). A part of the molecular complex of mouse salivary NGF is chemically similar to tissue kallikrein (Fiedler and Fritz, 1981), β-NGF endopeptidase and EGF-binding protein (Bothwell *et al.*, 1979). NGF is involved in accelerating the rate of wound healing in sialoadrenalectomized animals and the process is thought to be partially via a licking process (Li *et al.*, 1980). Schwab *et al.* (1976) suggested that NGF might function in protease inactivation during storage or protease synthesis, unrelated to effects on nervous tissue. Despite uncertainties in the physiology of NGF and other growth factors, their sources have served as aids in chemical characterization of the complexes.

Concentrations of both kallikrein and arginine esterase A can be increased by testosterone in the rat submandibular gland (Chao *et al.*, 1982). The quantity of submandibular proteases is under androgenic control. A digestive role for these enzymes is difficult to imagine because of the large quantitative difference between glands of male or female mice or the absence of enzymes prior to sexual maturity.

The idea that salivary gland kallikrein is involved in functional hyperaemia is not new (Hilton and Lewis, 1956). Although several counter-arguments have been expressed which indicate that kallikrein is largely secreted into ducts rather than into the circulation (Schachter *et al.*, 1977) (see Results), movement of the enzyme towards the vasculature is also apparent. Ørstavik *et al.* (1980b) showed that kallikrein injected retrogradely into the rat submandibular ducts could pass into the circulation. Enzyme secretion into the interstitium may possibly be via

granule-like structures which immunostain for kallikrein at the base of duct cells (Ørstavik *et al.*, 1980a; Simson *et al.*, 1983). Kallikrein was also located in the interstitium and basement membrane of salivary gland ducts (Ørstavik *et al.*, 1975; Kimura and Moriya, 1984a). A role for kallikrein in local blood flow regulation was also suggested by Rabito *et al.* (1982), who concluded that kallikrein was secreted into interstitial and vascular compartments since there was an arterial-venous difference in kallikrein concentration, and by Ørstavik *et al.* (1982b), who found an increase in blood flow on nerve stimulation after increasing kinin levels with converting enzyme inhibitor.

Schmid and Heidland (1984) found that exocrine salivary kallikrein secretion is elevated in rats with hypertension, though a rise in blood pressure may not be the cause of enhanced kallikrein secretion in hypertensive rats.

From the mouse submandibular glands, a related group of proteases (A, B, C and D) was found by Schenkein *et al.* (1969), who suggested that limited proteolysis converted "A" to "D". These esteroproteases were also shown to cause differentiation in muscle tissue of chick embryos. Furthermore, several other salivary esteroproteases were thought to be involved in processing precursors of NGF and EGF (Berger and Shooter, 1977; Bothwell *et al.*, 1979).

The primary and striking luminal localization of kallikrein in salivary ducts suggests that kallikrein is secreted into saliva. It was suspected that kallikrein at the luminal surface released kinins, which could in turn increase the apical permeability to ions and thus indirectly promote ion transfer either into or out of the duct lumen (Simson *et al.*, 1983). Tonin was also suspected to play a role in ion transport in the submandibular gland via generation of angiotensin II (Zacharatos *et al.*, 1983). Curiously, a selective intracellular secretion mechanism occurs for the release of kallikrein and tonin, since different α and β agonists cause differential protease release (Zacharatos *et al.*, 1983).

B. Kallikreins, Nerve Growth Factor and Related Proteases in Male Sex Glands and Their Possible Roles

Results in this thesis show the presence of tissue kallikrein (CGK) in epithelial cells of prostate, and coagulating glands and NGF in epithelial cells of the prostate gland. These findings are similar to those of Schachter *et al.* (1978) for kallikrein, and of Shikata *et al.* (1984) for NGF. Other related factors such as EGF (Shikata *et al.*, 1984) or androgen-dependent esterases (Isaacs and Shaper, 1983) are synthesized, stored and secreted by prostatic tissue. Tissue kallikreins are also found in porcine (Fink *et al.*, 1979) and human seminal fluid (Mann *et al.*, 1980; Geiger and Clausnitzer, 1981; Fink *et al.*, 1985), which probably originates from prostate glands.

In the male genital tract, as elsewhere, kallikrein may function physiologically by producing kinin via action on the ubiquitously occurring kininogens (Geiger and Clausnitzer, 1981). A common function for kallikrein in male sex glands based on its ability to release kinin is unlikely for mammals generally since only in the guinea-pig sex glands does the arginine esterase possess the ability to release kinin (Barton *et al.*, 1973).

Seminal plasma esterase has several molecular similarities to tissue kallikreins though some important ones are lacking. For example, canine prostate esterase does not release a kinin (Dubé *et al.*, 1983). The major androgen-dependent protein accounts for over 90% of the total protein present. Absence of the protein in prostates from castrated animals implies androgen dependence for synthesis, and correlates well with the morphological atrophy of the epithelium, measured by loss of secretory granules (Isaacs and Shaper, 1983).

It was proposed that motility of human spermatozoa is regulated by a kallikrein-kinin system (Schill and Haberland, 1974). Systemic kallikrein treatment of human patients improved sperm motility, volume of semen, sperm concentration and spermatozoa motility. Kallikrein also enhances sperm penetration through cervical mucus (Schill *et al.*, 1974). Evidence suggests that during normal capacitation of mammalian spermatozoa, a glycoprotein coat on the sperm plasma membrane is removed or modified, perhaps by protease action, and acceleration of the

acrosome reaction of hamster spermatozoa by proteolytic enzymes such as kallikrein seems to be partly due to alteration of sperm counts (Shinohara *et al.*, 1985).

Possible roles of NGF and other growth factors may be unrelated to, but in addition to, their effects on neuronal tissue. In the prostate gland, since it is an exocrine organ like the salivary glands, it is reasonable to speculate that these substances have some role related to the reproductive system.

C. Gastrointestinal Kallikreins and Their Possible Roles

A large body of evidence supports the concept of kallikreins playing fundamental roles in transport of electrolytes, glucose, and amino acids in the gastrointestinal tract (Moriwaki *et al.*, 1977).

Cuthbert and Margolius (1982) found that kallidin enhances gut chloride secretion, involving a prostaglandin pathway. Kinin receptors were thought to regulate this process (Manning and Snyder, 1982) despite an apparent location on the basolateral surface of colon epithelial cells and in the lamina propria and despite the fact kinins only stimulated intestinal electrogenic chloride secretion when applied to the basolateral (serosal) side of tissue (Cuthbert and Margolius, 1982; Manning and Snyder, 1982). In the intestine, chloride secretion occurs in crypt cells but not in surface cells (Welsh *et al.*, 1982) and it may be that the inability of kinin to effect transport at the surface is due to its inability to penetrate into the crypts (Kirkland *et al.*, 1985). Crocker and Willavoys (1975) found that bradykinin could either stimulate or inhibit transepithelial water transfer. Different mechanisms were proposed since inhibitory effects depended on whether the kinin was added to the serosal or mucosal bathing solution. Kallikrein administration into the intestine enhances amino acid absorption, which suggests that gastrointestinal kallikrein is related to nutrient absorption (Moriwaki *et al.*, 1977; Moriwaki and Fujimori, 1981). Geiger *et al.* (1977) suggested that kinins may function in absorption, and possibly affect transport of glucose and amino acids in the gut.

In the kidney tubule, there is evidence for kinin-mediated transport at both apical and basolateral sides of the cell (Kauker, 1980). Cuthbert *et al.* (1985) also found kinin influenced transport at both surfaces of cultured tubule cells. In the toad urinary bladder (Orce *et al.*, 1980) and rat renal cortical cells (Margolius and Chao, 1980), kallikrein-like proteases were effective near the mucosal surfaces in stimulating sodium transport.

Our findings of the presence of kallikrein in goblet cells of the gastrointestinal tract raises the possibility that, along with mucus, they may have a cytoprotective role. Kinins produced by colonic kallikrein may also contribute to pathological conditions such as diarrhea associated with carcinoid tumours (Donowitz and Binder, 1975).

The observation of greater changes in whole small intestine blood flow after kallikrein administration when compared with saline injection suggests that tissue kallikrein may regulate intestinal blood flow (Overlack *et al.*, 1983). If, as Zeitlin (1970) suggested, the kinins which are dilators and inhibit peristalsis, are important in intestinal absorption, they may be major factors in controlling normal vasomotor tone, permeability and motility of the intestine. It is interesting that 5-hydroxytryptamine, a vasoconstrictor substance that stimulates peristalsis, is also found along the length of the gastrointestinal tract.

D. Interpretation of Immunocytochemical Findings

Cytological procedures are used in attempts to discern relationships between structure and function. Immunocytochemical localizations are the most widely used methods and their aim is to identify and characterize proteins in tissue by labelling the antibody-antigen complex, making it visible.

The introduction of fluorescein-labelled immune sera (Coons and Kaplan, 1950) was a breakthrough in investigations of specific cellular components by light microscopy. Analogous techniques are also useful for ultrastructural studies where, in principle, the electron microscope can resolve an antibody molecule which has reacted with its antigen. Singer and Schick (1961), using ferritin-labelled antibody, successfully localized antigen at the electron

microscope level, but found the disadvantage of impaired reagent penetration.

With the advent of enzyme-labelled antibodies and the development of the unlabelled antibody method of Mason *et al.* (1969) and the PAP method of Sternberger and his colleagues (Sternberger, 1979) came increased sensitivity in detecting small amounts of antigen. This method was used in this thesis, and it is widely used in localization of intracellular antigens. A further advancement in antigen detection also used in this thesis, was made by Hsu *et al.* (1981) with the avidin-biotin complex method. Superior results are generally obtained and are attributed to the formation of a large complex containing multiple peroxidase molecules. The ABC method has several advantages over the PAP method, including lower background stain, easily recognized reaction product, shorter incubation times, and higher dilutions of reagents which can be used.

The pre-embedding immunostaining method, used occasionally in this thesis and found to be unsuccessful, has several disadvantages over the post-embedding method. First, immunoglobulins are unable to adequately penetrate into well-fixed thick sections. Secondly, non-specific globulin binding and randomly oriented reaction product make interpreting positive results difficult. Thirdly, reagents often do not permeate membranes easily. The major advantage of post-embedding immunostaining techniques, apparent in these results, is that immunoperoxidase reactions take place on the surface of routinely prepared ultrathin sections where intracellular antigens are accessible.

A fundamental conclusion which can be drawn from these localization experiments is that for each tissue examined and for a given antigen, specifically tailored methods of preparation must be developed. Although a search for the ideal immunocytochemical fixative produced several good results, no one treatment was universally applicable.

Discrepancies in localizations have occurred for other antigens where different techniques produced different results. In the colon, ethanol fixation produces carcinoembryonic antigen localization in epithelial cells (Rognum *et al.*, 1980; Primus *et al.*, 1981; Lindgren *et al.*, 1982; Kaku *et al.*, 1983). Formaldehyde-fixed, pronase-treated sections also produce similar

results, but without pronase treatment the sections do not produce the expected stain (Rognum *et al.*, 1980). Other studies which used different methods localized the same or very similar antigens in mucous cells (Rogalsky, 1975; Huitric *et al.*, 1976). Localizations of NGF in specific subcellular compartments of duct cells of submandibular glands have also yielded contradictory results, but in this case the discrepancies were related to inexperience with new methods. By immunofluorescence, NGF was first associated with cytoplasm, secretory granules, basal parts of cells and nuclei (Levi-Montalcini and Angeletti, 1961; Goldstein and Burdman, 1965; Kumar, 1972). A different pattern was later observed by Schwab *et al.* (1976), who localized the antigen only in the apical secretory granules. Since basal granules and other organelles did not stain, it was postulated that the NGF antigen is physiologically converted from a proprotein to an immunoreactive NGF, probably under the influence of a protease. Using methods very similar to those used in this thesis, Hofmann and Drenekhahn (1981) demonstrated NGF antigenicity uniformly in all secretory granules. Possibly in some studies cell damage caused protease activity to expose antigenicity during tissue fixation as a post-mortem artefact.

Localization of tissue kallikrein to acinar cells of the exocrine pancreas or to β cells of the endocrine pancreas in different studies appears to depend both on the specificity of α kallikrein antibodies used and the manner in which pancreatic tissue is prepared. Acinar localization of kallikrein in the pancreas was achieved by Ørstavik *et al.* (1981a), who used ethanol as the primary fixative, but ole Moi-Yoi *et al.* (1979a), who used formaldehyde, found antigenicity in endocrine islet cells. Lack of staining in acinar cells may be ascribed to antigenic masking since protease digestion, which was not used in their studies, produces immunostaining in acini (Pinkus *et al.*, 1983). Unmasking of endocrine kallikrein by trypsin or pronase suggests that it is less accessible to antibody than exocrine kallikrein.

E. Possible Sources of Circulating Tissue Kallikrein

Tissue kallikrein, in an active or inactive form, has been detected in plasma (Geiger *et al.*, 1980; Rabito *et al.*, 1980, 1982; Lawton *et al.*, 1981; Shimamoto *et al.*, 1984). Although its exact role in plasma is unknown, Masferrer *et al.* (1985) suggested that it could influence blood circulation via its ability to generate kinins. In plasma, the enzyme is present but bound to several inhibitors of different molecular weights (Johansen *et al.*, 1984).

Sources of the immunoreactive enzyme in plasma have not been determined, though several organs may contribute kallikrein to the circulation. Pancreatic kallikrein, which is absorbed across the intestinal wall (Moriwaki *et al.*, 1974), may contribute to circulating enzyme, but not significantly, since plasma from animals that had undergone subtotal pancreaectomy did not differ in antigen levels from control plasma (Lawton *et al.*, 1981). The submandibular gland was suggested to be a major contributor to circulating tissue kallikrein when labelled enzyme injected into the gland's main duct was detected in venous blood (Ørstavik *et al.*, 1980b). Support for this idea also came from Lawton *et al.* (1981), who found a great reduction in circulating antigen levels after bilateral submandibular and sublingual gland excision. The presence of residual antigen after removal of these glands suggests that other sources of enzyme such as parotid glands, pancreas, intestine or kidney exist.

The kidney may deliver tissue kallikrein into the circulation since enzyme antigenicity is associated with basolateral infoldings of tubule cells (Figueroa *et al.*, 1984). It is released into renal lymph and it is found in venous effluent of isolated perfused kidneys (Roblero *et al.*, 1976). The kidney may also play a role in clearance or metabolism of tissue kallikrein since an increased enzyme activity in plasma of nephrectomized rats was observed (Masferrer *et al.*, 1985). Renal kallikrein may also arise from circulating tissue kallikrein (Fiedler and Gebhard, 1980).

V. CONCLUSION

In this thesis, several kallikrein-like enzymes sharing immunological properties were localized in gastrointestinal, respiratory and male reproductive tissues. Nerve growth factor was also localized in male sex glands. Techniques were developed that were highly successful, particularly those using semi-thin resin embedded tissue sections and the avidin-biotin complex method of immunostaining.

In the various tissues examined here, antigen localization only occurred in apical regions of secretory cells and in secretions, indicating that kallikrein is an exported protein, likely playing a role in extracellular secretions. This finding is largely, though not exclusively, supported by other studies. Exceptions include cases of the kidney (Vio *et al.*, 1983), the pancreas (ole Moi-Yoi, 1979) and salivary glands (Kimura and Moriya, 1984a), where antigens were also localized in areas suggestive of endocrine secretion. Indeed, the presence of tissue kallikrein in the circulation indicates that it must be derived from at least one of the tissues known to synthesize it, and likely the mechanism by which the antigen enters the circulation is by endocrine-like secretion. While this thesis does not supply evidence for any endocrine secretion of kallikrein or nerve growth factor in tissues studied, it does not claim that this type of secretion does not exist.

Kallikrein enzyme activity has been implicated by many studies in a variety of essential physiological functions. Gene specific probes have recently isolated 25-30 members of the kallikrein family having 75% amino acid sequence homology (Mason *et al.*, 1983). Since sequences differ only at the active site region, it is apparent that functional differences may exist for all of the enzyme gene family members. Because three of these enzymes, tissue kallikrein, EGF-binding protein and γ-NGF are associated with bioactive peptide processing, it seems likely that other members will be discovered in similar roles.

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